Physiological Significance of Sulfur in Growth and Metabolism of Mustard (*Brassica juncea*) Exposed to Salinity Stress

ABSTRACT

THESIS

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Physiological Significance of Sulfur in Growth and Metabolism of Mustard (*Brassica juncea*) exposed to Salinity Stress

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Abstract of the thesis submitted to the Aligarh Muslim University, Aligarh, India for the degree of Doctor of Philosophy in Botany.

The present thesis comprises of six chapters.

In Chapter 1 (Introduction) the importance of the research problem and justifications for the present work undertaken were explained.

Chapter 2 is the Review of Literature. It deals with the relevant literature on the aspects of salinity stress and the importance of sulfur (S) nutrition in the alleviation of salinity stress on various crop plants. The chapter has been divided into sections and sub-sections for better understanding of the work of other research workers reported in this field of study.

Chapter 3 (Material and Methods) gives details of the material used in the study and methodology adopted to determine various physiological, biochemical, growth and yield characteristics recorded in the experiments. In addition, relevant information on the plant sampling and experimental design has been mentioned.

Chapter 4 (Results) includes details of results obtained in the three experiments. Variation in ATP-sulfurylase activity and S accumulation capacity in mustard cultivars were recorded. Details of results obtained on physiological and biochemical processes occurring in low and high S accumulation capacity cultivars under salinity stress and response of these two cultivars to S application alone or in combination with salinity stress have been included. The data were statistically analyzed and level of significance was determined at $P<0.05$ using analysis of variance (ANOVA).

In Chapter 5, results have been discussed in the light of observations recorded and supported with the earlier findings, if available on the subject. Possible explanations of the data obtained have also been included to reach a conclusion and possible future prospects.

Chapter 6 presents the summary of the work reported in this thesis.

In the following pages a brief account of the importance of the study undertaken, results obtained, conclusion and proposed future research are given.

Importance of the study undertaken

Salinity is one of the most important abiotic factors and a widespread agricultural problem in semi-arid regions which renders fields unproductive and limits plant growth and productivity to a great extent (Banzai *et al.* 2002; Munns 2002; Khan 2003; Flowers 2004; Parida and Das 2005; Sharifia *et al.* 2007; Athar *et al.* 2009; Turkan and Demiral 2009). The major causes of the soil salinity are inappropriate irrigation and the use of saline irrigation water. Further, the agronomic problem of salinity is compounded by the relatively low salt tolerance of many of the major crop plants (Maas and Hoffman 1977). High concentration of soluble salts in the top soil layer is detrimental to profitable agriculture. In dry areas, salt concentration increases in the upper soil layer due to high evaporatory loss that exceeds precipitation. Apart from the precipitation, the chemical constituents of water may undergo further changes through process of exchange, adsorption, differential mobility, etc., and the net result of these processes invariably is to increase the concentration in respect of Na^+ and Cl^- ions in the ground water in relation to their concentrations as the water moves from humid to arid areas.

The problem of saline soils is increasing because of inadequate irrigation and drainage practices. As mentioned earlier the area of degraded land is increasing with varying degrees of salt accumulations. For sustainable crop production, it is important to develop methods or techniques for alleviating the NaCl-induced growth inhibition and reducing its accumulation in plants. Increasing evidence suggests that mineral-nutrient status of plants plays a critical role in increasing plant resistance to environmental stresses (Marschner 1995, Vassilev *et al.* 2005, Anjum *et al.* 2008a,b, Hassan *et al.* 2008a,b; Khan *et al.* 2008). The use of S opens potential option which can be used to alleviate salinity stress. Sulfur is a structural constituent of several coenzymes and prosthetic groups, such as ferredoxin, which are also important for N assimilation. Thus, S plays an important role in plant growth and in the regulation of plant development (Ernst *et al.* 1998; Nazar *et al.* 2008) and required in higher quantity by mustard (Zhao *et al.* 1993, Lakkineni and Abrol 1994, McGrath and Zhao 1996). It is a precursor of cysteine and methionine, amino acids involved in the synthesis of compounds containing reduced S (Marschner 1995, Scherer 2001). A sufficient S supply improves photosynthesis and growth (Ahmad *et al.* 2005, Khan *et al.* 2005) through regulating N

assimilation (Reuveny *et al.* 1980, Kopriva *et al.* 2002, Scherer 2008). A large accumulation of N maintains high chlorophyll content and high activity of enzymes of Calvin cycle (Lawlor *et al.* 1989), and enhances growth (Schnug *et al.* 1993, Khan *et al.* 2005) because of the established role of S and N in cell differentiation and overall growth of plants (Marschner 1995). The assimilatory pathways of S and N have been considered functionally convergent and well coordinated as the availability of one element regulates the other (Reuveny *et al.* 1980, Schnug *et al.* 1993). The availability of S regulates the activity of nitrate reductase and the accumulation of N (Pal *et al.* 1976).

The oleiferous *Brassica* is the third most important source of vegetable oil in the world after palm and soybean oil and grown as an edible or an industrial oil crop which is used as a source of edible protein, in much the same way as soybean protein (Zhang *et al.* 2003). *Brassica* is the Latin name of a genus that is taxonomically placed within the Brassicaceae (Cruciferae), which is one of the tenth most economically important plant families in the world. The major mustard oil-producing countries include Canada, China, France, Germany, India and UK. According to a report of the United States Department of Agriculture (USDA), the world oilseed production was 397metric tons in 2006-07. Indian agriculture contributes about 15% and 8% to the world total acreage under oilseed cultivation and production, respectively. However, the productivity in India is only 791kg/ha as compared to the world average of 1718kg/ha (Damodaran and Hedge 2002). About 90% of the total land under oilseed cultivation in India is occupied by *Brassica juncea* (Khan *et al.* 2007).

Discussed the importance of sulfur assimilation in plant metabolism and their role in stress tolerance it was assumed that mustard (*Brassica juncea* L. Czern & Coss.) may serve as a model crop in the study of the influence of S in plant metabolism under salinity stress. Further, the fertilization of crop with balanced S may prove fruitful in alleviating salinity stress and improving crop productivity. Studies on crops with a view of alleviating salinity stress with balanced S application have not been carried out. Therefore, the reported research was undertaken with the following objectives:

- To screen and select the S-efficient and S-inefficient mustard cultivars by studying the activity of ATP-sulfurylase enzyme and S accumulation.
- To study the physiological response of high ATP-sulfurylase activity and low ATP-sulfurylase activity cultivars of mustard to salinity stress.

- To study the influence of sulfur in the alleviation of salinity stress-induced adverse effects in mustard cultivars with high and low ATP-sulfurylase activity.

Experimental Results

The results of the experiments are summarized below:

Experiment 1

Experiment 1 was conducted on four mustard (*Brassica juncea* L.) cultivars namely, Alankar, Varuna, Pusa Jai Kisan and SS2 to select S-efficient and S-inefficient cultivars on the basis of ATP-sulfurylase activity and S accumulation. In addition, sulphate content (SO_4^{2-}) in root and leaf, N content in leaf, growth, photosynthetic characteristics at 30 and 60 DAS were also recorded. Sulfate transport index (STI) was calculated as the ratio of sulfate content in root and leaf and expressed as percentage. The relationship of ATP-sulfurylase activity with photosynthetic rate and shoot dry mass was also established. The treatments were arranged in a randomized block design and replicated three times. All the cultivars differed in ATP-sulfurylase activity and sulfate transport index. Pusa Jai Kisan showed maximum ATP-sulfurylase activity and STI followed by Alankar, Varuna and SS2. Leaf sulfate, nitrogen content, photosynthetic rate, leaf area and shoot dry mass were also increased from 30 to 60 DAS. A strong positive correlation ($P<0.01$) between ATP-sulfurylase activity and photosynthetic rate and shoot dry mass was found in all the four cultivars. It was concluded that the activity of ATP-sulfurylase may be used as a physiological trait for augmenting photosynthesis and shoot dry mass accumulation in mustard.

Experiment 2

Experiment 2 was conducted on the basis of findings of Experiment 1. As observed in Experiment 1, Pusa Jai Kisan emerged as S-efficient and SS2 as S-inefficient mustard cultivars. This experiment was conducted with the aim of studying the influence of 0, 50 and 100 mM NaCl on sulfur assimilation, photosynthetic traits, water relations, contents of nutrients and ions, oxidative stress, various components of antioxidant defense system and growth in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (low ATP-sulfurylase activity) cultivars of mustard at 30 and 60 DAS, and yield characteristics at 120 DAS. The experiment was conducted in a factorial randomized block design and each treatment was replicated three times. Maximum reductions in the growth, photosynthetic characteristics and nutrients content were noted with 100 mM NaCl at 30 and 60 DAS in both the cultivars of the mustard. Plants treated with 100 mM

NaCl exhibited a significant and maximum decrease in the characteristics over control. The activity of ATP-sulfurylase in plants grown with NaCl was significantly higher than the control in both the cultivars. Pusa Jai Kisan exhibited higher ATP-sulfurylase activity than SS2 at both NaCl levels. However, the effect of 50 and 100 mM NaCl on ATP-sulfurylase activity did not differ significantly in both the cultivars. The cultivar Pusa Jai Kisan exhibited high capacity of accumulating Na^+ and Cl^- in root than leaf. Contrarily, SS2 showed higher content of Na^+ and Cl^- in leaf with lower content in root. The higher level of Na^+ and Cl^- in leaf in SS2 induced greater oxidative stress affecting membrane permeability more adversely than in Pusa Jai Kisan causing greater reductions in photosynthesis and plant dry mass. Pusa Jai Kisan exhibited lower induction of superoxide dismutase activity but higher induction of catalase, ascorbate peroxidase and glutathione reductase in comparison to SS2. The lower superoxide dismutase activity in Pusa Jai Kisan was due to the lower content of leaf Na^+ and Cl^-. The enhanced activity of ATP-sulfurylase and glutathione reductase and glutathione content resulted in lower content of TBARS and H_2O_2 in Pusa Jai Kisan. This cumulatively resulted in lesser reductions in photosynthetic functions in Pusa Jai Kisan. The cultivar Pusa Jai Kisan with high ATP-sulfurylase activity showed greater tolerance to salinity stress as the result of its capacity to accumulate Na^+ and Cl^- in root, higher water and osmotic potential, efficient antioxidant system and higher glutathione content. The increased activity of antioxidant enzymes and glutathione in this cultivar removed reactive oxygen species more efficiently. These characteristics of Pusa Jai Kisan helped in protecting the photosynthetic capacity and maintaining high plant dry mass. Contrarily, SS2 cultivar with low ATP-sulfurylase activity had higher accumulation of Na^+ and Cl^- in leaf, lower water potential and osmotic potential, lower contents of nutrients, greater oxidative stress and poor capacity of antioxidant system resulting in lower photosynthesis and dry mass than Pusa Jai Kisan. Salinity stress caused significant reduction in yield and its attributes at harvest and the extent of decrease was greater in SS2 than Pusa Jai Kisan with 100 mM NaCl treatment.

Experiment 3

Experiment 3 was conducted to study the influence of 1 or 2 mM SO_4^{2-} in the alleviation of 100 mM salinity stress in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (low ATP-sulfurylase activity). Plants were raised with 0, 100 mM NaCl, 1 mM SO_4^{2-}, 2 mM SO_4^{2-}, 100 mM NaCl + 1 mM SO_4^{2-} and 100 mM NaCl + 2 mM SO_4^{2-} to

observe response on sulfur assimilation, photosynthetic traits, water relations, contents of nutrients and ions, oxidative stress, various components of antioxidant defense system and growth at 30 and 60 DAS, and yield characteristics at harvest. The treatments were arranged in a factorial randomized block design and each treatment was replicated three times.

Salinity stress led to a significant reduction in photosynthetic traits, water potential and osmotic potential, nutrients content and yield characteristics of both the cultivars. The low ATP-sulfurylase activity cultivar SS2 exhibited a higher reduction than high ATP-sulfurylase activity cultivar Pusa Jai Kisan. In comparison to 1 mM SO_4^{2-}, the application of 2 mM SO_4^{2-} maximally alleviated the adverse effects of 100 mM NaCl and improved the photosynthetic traits, water and osmotic potential, nutrients content, components of enzymatic and non-enzymatic antioxidant system, growth and yield characteristics of both the cultivars, but to a greater extent in Pusa Jai Kisan than in SS2. In general, under non-stressed condition, application of 1 mM SO_4^{2-} was found more effective than 2 mM SO_4^{2-} in improving photosynthesis, water relations, plant growth, antioxidant metabolism, growth and yield in both the cultivars. It is pertinent to mention here that the application of sulfur either alone or in combination with 100 mM NaCl further increased the ATP-sulfurylase activity and S content in both the cultivars, which were found greater in Pusa Jai Kisan than SS2.

Conclusion

Conclusively, nutrients are known to play an essential role in plant metabolism and augmenting growth and productivity of crops. On the basis of ATP-sulfurylase activity and S accumulation Pusa Jai Kisan found to be S-efficient and SS2 as S-inefficient cultivar. Therefore, Pusa Jai Kisan exhibited lesser decrease in photosynthetic traits, nutrients content and growth characteristics. Correspondingly, Pusa Jai Kisan also showed lesser oxidative stress and increased in nutrients content and antioxidant system than SS2 to protect photosynthetic machinery and consequent effects on other attributes when exposed to 100 mM NaCl. The salt treatment caused an accumulation of Na^+ and Cl^- ion. Sulfur proved significant potential in the alleviation of NaCl stress in both the cultivars. The decreases in the characteristics observed due to NaCl stress were lowered by S application and 2 mM SO_4^{2-} proved more effective in alleviation of NaCl stress than 1 mM SO_4^{2-}. The application of 1 mM SO_4^{2-} instead of 2 mM SO_4^{2-} was found positive in improving photosynthesis, water relations, plant growth, antioxidant metabolism, growth

and yield in both the cultivars. However, when applied in combination with 100 mM NaCl, higher dose of S (2 mM SO_4^{2-}) was found effective in alleviating the 100 mM NaCl-caused effects in both the cultivars but to a great extent in high ATP-sulfurylase activity cultivar Pusa Jai Kisan compared to low ATP-sulfurylase activity cultivar SS2. The cultivar Pusa Jai Kisan responded more to S application in the alleviation of NaCl stress than the SS2, which could explain the ability of S-efficient and salt tolerant cultivar to show better yield under salinity stress. It may be suggested that the application of 2 mM SO_4^{2-} may be used for alleviating the salinity stress and to obtain higher yield under salinity stress.

Future Research

Salinity is the major environmental factor limiting plant growth and productivity in the arid and semi-arid regions of the world. The continuous accumulation of salt in cultivated soils as a result of irrigation and climate warming increases the importance of the study of this stress factor. Salinity stress causes an osmotic inhibition and ionic toxicity, which affects the physiological and biochemical functions of the plant cell. The present study showed that the maximum reduction in the growth, photosynthetic, biochemical and yield characteristics was noted with 100 mM NaCl in both the cultivars of mustard, i.e., Pusa Jai Kisan and SS2. However, the reduction in the characteristics was much pronounced in SS2. Mineral nutrient status plays an important role in increasing plant resistance to environmental stress factors. Of the mineral nutrients, S constitutes one of the macronutrient necessary for the plant life cycle. The processes of the uptake and assimilation of S in higher plants are crucial factors determining plant growth and vigor, crop yield and the resistance to biotic and abiotic stresses. Sulfur assimilates not only play key roles in the primary metabolism of plants and provide structural components of essential cellular molecules, but also act as signaling molecules for cellular communication with the environment. The application of sulfur on ATP-sulfurylase activity and sulfur assimilation, photosynthetic traits, biochemical characteristics, oxidative stress, antioxidant system, growth and yield characteristics under NaCl stress has been reported for the first time. Efforts have been made to develop S protocol for the alleviation of NaCl stress in mustard.

Future strategies are focused to modulate steps of S assimilation pathways leading to the production of thiols and their products in plants through manipulating serine acetyl transferase (SAT), γ-glutamyl- cysteine synthetase and glutathione synthetase enzymes

under salinity stress. More detailed studies are required to understand the NaCl-induced stress response modulated by S metabolism at physiological and molecular levels. This would help to develop an effective strategy to raise transgenic species for NaCl resistance.

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Dedicated to My Family
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CERTIFICATE

This is to certify that the thesis entitled, **Physiological significance of sulfur in growth and metabolism of mustard (*Brassica juncea*) exposed to salinity stress** submitted for the degree of **Doctor of Philosophy** in **Botany** is a faithful record of bonafide research work carried out at the **Aligarh Muslim University, Aligarh** by **Ms. Rahat Nazar** under my guidance and supervision and that no part of it has been submitted for any other degree or diploma.

(Nafees A. Khan)

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ABBREVIATION

%	Percent
$.^1O_2$	Singlet oxygen
°C	Degree Celcius
Acetyl CoA	Acetyl Coenzyme A
AMP	Adenosine monophosphate
APK	APS kinase
APR	APS-reductase
APS	Adenosine 5'-phosphosulfate
APX	Ascorbate per oxidase
AsA	Ascorbate
ATP-s	ATP-sulfurylase
BC	Before Christ
Ca	Calcium
CAT	Catalase
Cl^-	Chloride ion
cm	Centimeter
cv	Cultivar
Cys	Cysteine
DAS	Days after sowing
DHA	Dehydroascorbate
dSm^{-1}	Deci Siemens per metre
DTNB	5', 5'-dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
EC	Electrical conductivity
EDTA	Ethylenediamine tetraacetic acid
Fe-SOD	Iron superoxide dismutase
Fv/Fm	Chlorophyll fluorescence
g	Gram
GPOX	Guaiacol peroxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S transferase
h	Hour
H_2O_2	Hydrogen peroxide
ha	Hectare
K	Potassium
LPO	Lipid peroxidation
LSD	Least Significant Difference
MDA	Monodehydroascorbate
Meq	Milliequivalent
Mg	Magnesium
mg	Milligram
min	Minute
ml	Millilitre

mM	Milli molar
MPa	Mega pascal
MSI	Membrane Stability Index
N	Nitrogen
Na^+	Sodium ion
Na_2ATP	Disodium adenosine triphosphate
Na_2EDTA	Sodium ethylenediamine tetraacetic acid
Na_2MoO_4	Sodium molybdate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NEM	N'N-ethylemaleimide
nm	Nanometer
nmol	Nanomole
$O_2^{\cdot-}$	Superoxide anion radicals
OAS	O-acetylserine
OAS-TL	O-acetylserine(thiol) lyase
OH	Hydroxyl radical
P	Phosphorus
PAPS	Phosphoadenosine 5`-phosphosulfate
pH	Hydrogen ion
PNS	Plant nutrient sulfur
POX	Peroxidase
ppm	Parts per million
PSI	Photosystem I/ Pigment system I
PSII	Photosystem II/ Pigment system II
PVP	Polyvinylpyrrolidone
RFLP	Restriction fragment length polymorphism
RGR	Relative growth rate
ROS	Reactive oxygen species
RSI	Relative salt injury
S	Sulfur
S^{2-}	Sulfide
SAR	Sodium absorption ratio
SAT	Serine acetyl transferase
SE	Standard error
Ser	Serine
S-H	Sulfhydryl
SiR	Sulphite reductase
SO_3^{2+}	Sulphite
SO_4^{2-}	Sulphate
SOD	Superoxide dismutase
STI	Sulfate transport index
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic acid
µmol	Micromole
Ψ_w	Water potential
Ψ_τ	Osmotic potential

CONTENTS

Introduction

INTRODUCTION

Environmental factors and their interactions are important in determining performance and distribution of plants. In agricultural system, the environmental factors influence agricultural productivity and also determine quality of the produce. Soil salinity is one of the major environmental abiotic factors reducing plant growth and productivity worldwide (Allakhverdiev *et al.* 2000). In arid and semi arid regions of the world desertification, land degradation and declining precipitation rates increasingly limit area for crop cultivation (Choukr-Allah and Harrouni 1996). It has been estimated that soil salinization affects about 7% of the global total land area (Glenn *et al.* 1998; Koyro *et al.* 2008) and 20-50% of the global irrigated farmland (Tanji 2002; Hu and Schmidhalter 2005; Koyro *et al.* 2008). The latter is mostly affected by secondary salinization caused by human interference such as irrigation, deforestation, overgrazing, or intensive cropping (Ashraf 1994; Pitman and Läuchli 2002; Türkan and Demiral 2008; Geissler *et al.* 2009). Salinization also results in further shortage of the already limited freshwater resources (Lieth and Moschenko 1998; Hamdy 2002). Simultaneously, as the world population is presently increasing by 80 million people every year (Deutsche Stiftung Weltbevölkerung 2008), the requirement of the cultivable farmland and of fresh water continuously increases. At present, its extent throughout the world is increasing regularly (Schwabe *et al.* 2006) and it has now become a very serious problem for crop production (Munns and Tester 2008; Khan *et al.* 2009b; Palma *et al.* 2009). However, the intensity of salinity stress varies from place to place.

Salinity of soil and water is caused by the presence of excessive amounts of salts. Most commonly, high amounts of Na^+ and Cl^- cause the salinity stress. Salinity stress has deleterious effects on plant growth are associated with (*i*) low water potential of soil solution (water stress), (*ii*) nutritional imbalance, (*iii*) specific ion effect (salt stress), or (*iv*) a combination of these factors (Ashraf 1994; Marschner 1995). All of these factors cause adverse pleiotropic effects on plant growth and development at cellular (Levitt 1980; Gorham *et al.* 1985; Munns 2002) and at the molecular level (Winicov 1998; Mansour 2000; Tester and Davenport 2003). Finally, the detrimental effect of high salinity on plants is observed at the whole plant level as death of plants

and/or decrease in the productivity. Salt stress adversely affects plant metabolism, thus influencing all major processes such a growth, photosynthesis, water relations, protein synthesis and lipid metabolism.

Reviews are available describing the effects of salinity stress on plant growth and metabolism (Parida and Das 2005; Manchanda and Garg 2008). High concentrations of salt disrupt homeostasis in water potential and ion distribution in plants. Altered water status most likely brings about initial growth reduction (Dash and Panda 2001). Crucial changes in water and ion homeostasis lead to molecular damage, growth arrest and even death. Specific effects of salt stress on plant metabolism have been related to the accumulation of toxic ions (Na^+ and Cl^-) or to the depletion of K^+ and Ca^{2+} (Kingsburry and Epstein 1986; Rengel 1992; Pérez-Alfocea *et al.* 1996; Al-Karaki 2000). As a consequence of ion imbalance, which is primary effects of salt stress, secondary stress such as oxidative damage may occur. Limited CO_2 fixation due to salt stress conditions leads to the decrease in the availability of oxidized $NADP^+$ to serve as an electron acceptor in photosynthesis. With the over reduction of ferredoxin during photosynthetic electron transfer, electrons are transferred from PS I to oxygen to form superoxide radicals ($O_2^{\cdot-}$) by the process of Mehler reaction, which triggers chain reactions that generate more destructive oxygen radicals (Polle 1996; Hsu and Kao 2003). It is already known that cytotoxic reactive oxygen species (ROS) are constantly generated during metabolic processes in the mitochondria, peroxisomes and cytoplasm and can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acid (McCord 2000).

Photosynthesis is the process that determines plant growth and vigour. If the process is affected the whole of the metabolism gets disrupted due to non-availability of metabolites. Photosynthesis involves a long chain of mechanisms, enzymes, and intermediate products and is regulated by several external and internal factors. Photosynthetic efficiency depends on the sequence of metabolic events such as photochemical reactions, on the enzymes involved in carbon assimilation, on the structure of the photosynthetic apparatus, and on the transport of photosynthetic intermediates (Parida and Das 2005). Photosynthetic capacity of different plant species is suppressed by salinity stress (Robinson *et al.* 1983; Ball and Farquhar 1984; Seeman

and Critchley 1985; Bowman and Strain 1987; Dubey 1997; Makela *et al.* 1999). The reduction in photosynthesis under saline conditions may be due to (*i*) reduced conditions of CO_2 intake by the photosynthetic organs, (*ii*) modification in the structure and function of photosynthetic organelles, (*iii*) alterations in light reactions and/or (*iv*) reduced rate of transport of assimilates and intermediary compounds to the metabolic sinks. Salinity affects chlorophylls, leading to a decreased absorption of light by the chloroplasts and thus indirectly impairing photosynthesis. The magnitude of reduction in chlorophyll (Chl) a and Chl b as well as in total chlorophyll by salinity is determined by the absolute concentration of chloride and/or sodium ions in the leaves (Khan 2003; Grattan and Grieve 1999; López-Climent *et al.* 2008). Suwa *et al.* (2006) and Tagaki *et al.* (2009) reported that adverse effect of salinity on plant biomass production was due to impairment of sink activity earlier than source activity. In other words, sink activity is depressed earlier by salinity stress and then photosynthate transport is impaired leading to sugar accumulation causing decreased photosynthetic activity of leaves.

As plants are more exposed to environmental factors, they are more prone to oxidative damage (Foyer *et al.* 1994). The oxidative damage enhances lipid peroxidation and alters functionality of membranes, and causes changes in enzymatic and non-enzymatic antioxidant systems (Khan *et al.* 2007a; Mobin and Khan 2007; Anjum *et al.* 2008a; Khan *et al.* 2009b). Therefore, there is a constant need for efficient mechanism/s to compensate the possible oxidative damage caused to cellular components by oxidative damage. Plants develop number of strategies to cope with the adverse effects of salt stress. These strategies may be: (i) selective accumulation of nutrients or exclusion of ions, (ii) control of ions uptake by roots and their transport to leaves, (iii) compartmentalization of ions at the cellular and whole plant leve, (iv) synthesis of compatible osmolytes, (v) alteration in photosynthetic pathway, (vi) changes in membrane structure, (vii) induction of antioxidative enzymes, and (ix) induction of phytohormones (Parida and Das 2005).

Among antioxidant enzymes, superoxide dismutase (SOD; EC 1.15.1.1) constitutes the primary step of cellular defense and dismutates superoxide radicals ($O_2^{\cdot -}$) to H_2O_2 and O_2. Accumulation of H_2O_2, a strong oxidant, is prevented in the cell either by catalase (CAT; EC 1.11.1.6) or by the ascorbate-glutathione cycle, where

ascorbate peroxidase (APX; EC 1.1.11.1) converts it to H_2O. Finally, glutathione reductase (GR; EC 1.6.4.2) catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Noctor *et al.* 2002). Ascorbate (AsA) and GSH are important components of ascorbate-glutathione cycle responsible for the removal of H_2O_2 in different cellular compartments (Foyer *et al.* 2002). Ascorbate is the major, probably the only, antioxidant buffer in the apoplast and is an essential metabolite involved with vital cell functions. It is a primary key antioxidant that reacts directly with $OH^{\cdot}$ radicals, $O_2^{\cdot -}$, and $_1O_2$ (Chen and Gallie 2004). Glutathione present in plant cells is a major non-enzymatic scavenger of ROS (Mishra *et al.* 2006). In particular, GSH plays an important role in response to various biotic and abiotic stresses as it is a major thiol-disulfide redox buffer in plant cells (May *et al.* 1998a; Schafer and Buettner 2001) and is dependent on the availability of cysteine, which in turn is dependent on sulfate assimilation. For this reason, its concentration is controlled by a complex homeostatic mechanism where the availability of sulfur (S) seems to be required (May *et al.* 1998a, b). Both AsA and GSH can also react directly and scavenge certain ROS.

Among various strategies, nutrient management may be an important aspect for alleviating stress and optimizing yield under varied environmental conditions. Sulfur is an essential macronutrient and occupies fourth place in importance after nitrogen (N), phosphorous (P) and potassium (K). It plays a pivotal role in the regulation of plant growth and development under normal and stress conditions (Ernst 1998; Nazar *et al.* 2008). The uptake and assimilation of S in higher plants form important aspects in determining crop yield, quality and resistance to environmental stresses. In a cascade of enzymatic steps, inorganic S is converted to metabolically important S containing compounds like cysteine, methionine, S-adenosyl methionine, glutathione, co-enzymes, thioredoxins, sulpholipids and vitamins such as biotin, thiamine and ferredoxin (Hell 1997; Hell and Rennenberg 1998; Saito 2000). Sulfur is an important in the formation of sulfhydryl (S-H) and disulphide bonds (S-S). These bonds are important for the stabilization of protein structure. In spite of the several-fold importance of the element, the attention to use this element as a major agricultural fertilizer has been ignored due to priority on the use of N, P and K. This has resulted in the deficiency of S in

agricultural soils over the past several years (Scherer 2001). Among different regions, Asia has the highest S fertilizer requirement. Among Asian countries, India and China together currently account for about 60% of the total estimated S deficit. Continuous mining of S from soils has led to widespread S deficiency and negative soil budget (Aulakh 2003). One of the main reasons for limited research on this important nutrient has been attributed to the difficulty in measuring S in soils (Tillman 1988). It is now widely recognized that S deficiency results in yield losses and quality deterioration through its own effect and through effect on N assimilation.

Sulfur assimilation has been reported to be tightly linked with plant tolerance to a number of abiotic stress factors (Anjum *et al.* 2008a; Khan *et al.* 2009a). The first step of S assimilation in plants is the activation of S by the enzyme ATP-sulfurylase. It is the rate-limiting enzyme that regulates the biosynthesis of non-protein thiol glutathione and in turn also enzymes of ascorbate-glutathione pathway (Tausz *et al.* 2004; Khan et al. 2009b; Szalai *et al.* 2009) (Figure 1). Therefore, higher expression of ATP-sulfurylase activity is necessary for the maintenance of optimal GSH levels required for the proper functioning of ascorbate-glutathione cycle (Khan *et al.* 2009a, b). Glutathione has been shown to take part in the removal of excess H_2O_2 (Noctor and Foyer 1998) and lipid peroxidases (Rausch *et al.* 2007; Srivalli and Khanna-Chopra 2008). It appears logical to consider that crops with high S requirement and capacity for its assimilation will have greater GSH synthesis and tolerance to abiotic stress than crops with reduced S assimilation.

The oleiferous *Brassica* is the third most important source of vegetable oil in the world after palm and soybean oil. It is grown as an edible or an industrial oil crop used as a source of edible protein, in much the same way as soybean protein (Zhang *et al.* 2003). *Brassica* is the Latin name of a genus that is taxonomically placed within the Brassicaceae (Cruciferae), which is one of the tenth most economically important plant families in the world. The major mustard oil-producing countries include Canada, China, France, Germany, India and UK. According to a report of United States Department of Agriculture (USDA), the world oilseed production was 397 metric tons in 2006-07. Indian agriculture contributes about 15% to the world total acreage under oilseed cultivation and 8% to the production, but the productivity in India is only 791

kg/ha as compared to the world average of 1718 kg/ha (Damodaran and Hedge 2002). *Brassica juncea* occupies about 90% of the total land under oilseed cultivation in India. It has high S requirement compared to other crops (Khan *et al.* 2007b).

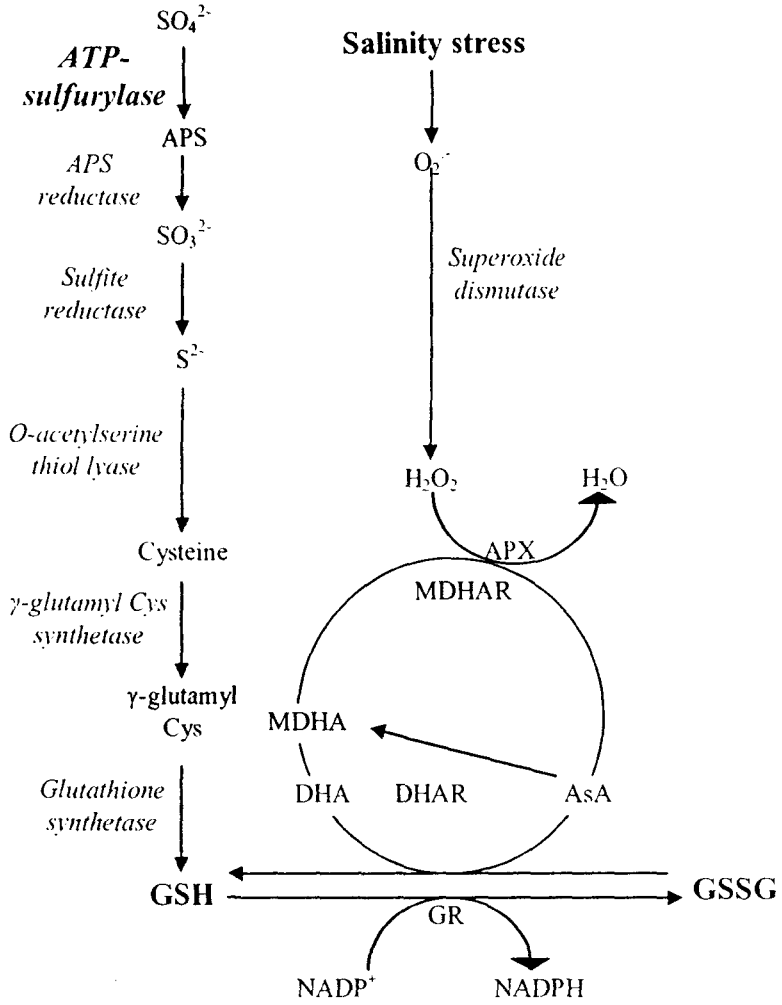


Figure 1. Relationship of ATP-sulfurylase with glutathione and components of ascorbate-glutathione pathway (APS: adenosine 5-phosphorulfate; APX: ascorbat peroxidise; AsA: asocrbate; DHA: dehydroascorbate; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; MDHA: monodehydroascorbate; MDHAR: monodehydroascorbate reductase) (published as Khan *et al.* 2009b).

In view of the importance of S in crop growth and metabolism, it is assumed that mustard (*Brassica juncea* L. Czern & Coss.) may serve as a model crop in the study of the influence of S on plant metabolism under salinity stress. It was postulated that mustard types that differ in S assimilation (determined on the basis of ATP-sulfurylase activity) would show different degree of tolerance to salinity stress, and S application might prove helpful in alleviating salinity stress. Sulfate reduction is maximal during leaf development (expansion) but declines rapidly after maturation.

Keeping these aspects in mind the research reported in this thesis was undertaken with the following objectives:

- To screen and select the S-efficient and S-inefficient mustard cultivars by studying the activity of ATP-sulfurylase enzyme and S accumulation.
- To study the physiological response of high ATP-sulfurylase activity and low ATP-sulfurylase activity cultivars of mustard to salinity stress.
- To study the influence of sulfur in the alleviation of salinity-induced adverse effects in mustard cultivars with high and low ATP-sulfurylase activity.

Review of Literature

Contents

REVIEW OF LITERATURE

2.1 Introduction

Agriculture is one of the oldest accomplishments man has made after learning the art of hunting animals with spear and bow. This practice probably began in the Mesolithic or Middle stone age from 12000 to 6000 BC. Later, they also advanced their knowledge to improve productivity of crops to feed every mouth. In the successful era of Green Revolution efforts were made to increase productivity of crops with the involvement of multiple factors. The primary focus in increasing crop productivity was the use of inorganic fertilizers, irrigation and high yielding cultivars. In an effort of achieving the goal of enhancing crop productivity with the use of chemical fertilizers and waste water irrigation a variety of abiotic stress factors limiting crop productivity have been added. This is in addition to the already existing deteriorated soil. Salinity of soil is a major abiotic stress factor. It is the first chemical stress factor encountered during the evolution of life on earth because earliest living organisms were marine forms (Jacobsen and Adams 1958).

Salinity is one of the most important abiotic stress factors and a widespread agricultural problem in semi-arid regions which renders fields unproductive and limits plant growth and productivity to a great extent (Banzai *et al.* 2002; Munns 2002; Khan 2003; Flowers 2004; Parida and Das 2005; Sharifia *et al.* 2007, Athar *et al.* 2009; Turkan and Demiral 2009). Salinization is the increase in concentration of total dissolved solids in the soil. Soils are classified as saline when the electrical conductivity (EC) is 4 dS/m or more (USDA-ARS 2008), which is equivalent to approximately 0.2 MPa. This definition of salinity derives from the EC that significantly reduces the yield of most crops (Munns and Tester 2008).

Salinity is detrimental to plants as it causes various kinds of alterations such as (i) ion toxicity (ii) nutritional constraints by decreased uptake of N, P, K and Ca and (iii) water and osmotic stress: Na^+ competes with K^+ in biochemical reactions. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the non-covalent interactions among amino acids of proteins. This leads to conformational changes and loss of protein functions. In addition, ion toxicity, osmotic

stress and nutritional defects under salinity may lead to metabolic imbalances causing oxidative stress (Zhu 2001).

2.1 Salt-affected Soils

Salt-affected soils occur in all continents and under almost all climatic conditions. Their distribution, however, is relatively more extensive in the arid and semi-arid regions compared to the humid regions. Over 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated conditions of sodicity (434 million ha) (Munns 2005; FAO 2008). This is over 6% of the world's total land area. However, a significant proportion of recently cultivated agricultural land has become saline because of land clearing or irrigation. Of the 1500 million ha of land farmed by dry land agriculture, 32 million (2%) are affected by secondary salinity to a varying degrees. Of the current 230 million ha of irrigated land, 45 million ha, are salt affected (20%) (FAO 2005). Irrigated land is only 15% of total cultivated land, but as irrigated land has at least twice the productivity of rain-fed land, it produces one-third of the world's food. Increased drought and salinization of arable land are expected to have devastating global effects (Wang *et al.* 2003). It was estimated that the current amount of annual loss of arable area could double by the end of the century because of global warming (Evans 2005).

About 7-12 million ha of land in India are known to have been degraded by salinity with varying degrees of salt accumulation. This problem is acute in the semi-arid and arid tracts of Indo-Gangetic alluvial plains where about 40% of the total salt-affected area is concentrated (Agarwal *et al.* 1979). Besides, an additional area of about 15-20 million ha of land in canal irrigated tracts runs the risks of being degraded through the influence of salts (Abrol 1986). The major causes of the soil salinity are inappropriate irrigation and the use of saline irrigation water. In dry areas, salt concentration increases in the upper soil layer due to high evaporatory loss that exceeds precipitation. Apart from the precipitation, the chemical constituents of water may undergo further changes through process of exchange, adsorption, differential mobility, etc., and the net result of these processes invariably is to increase the concentration in respect of Cl^- and Na^+ ions in the ground water in relation to their concentrations as the water moves from humid to arid areas.

2.2 Classification of Salt-affected Soil

In the course of accumulation of knowledge on characteristics and plant growth relationships in salt affected soils, two main groups of these soils have been distinguished (Szabolcs 1974). These are:

2.2.1 Saline soils

Soils containing sufficient neutral soluble salts adversely affect the growth of most crop plants. The soluble salts are chiefly sodium chloride (NaCl) and sodium sulphate ($NaSO_4$). But saline soils also contain appreciable quantities of chlorides and sulphates of Ca and Mg. This group of soils is both saline and alkali. They have appreciable amounts of soluble salts as indicated by the values of EC which are > 4 dS/m. Also, the exchangeable Na^+ percentage is greater than 15. The pH, however, is likely to be less than 8.5.

2.2.2 Sodic soils

Soils containing sodium salts capable of alkaline hydrolysis mainly as Na_2CO_3 have been termed as 'Alkali' in older literature. These soils do not contain any large amount of neutral salts and as such the EC is < 4 dS/m. The detrimental effect of alkali soil on plants is largely due to the toxicity of high amount of exchangeable sodium and pH. Alkali soils have an exchangeable sodium percentage of more than 15 and pH greater than 8.5. Such soils have low infiltration rate and the physical condition is unfavorable. Because of high alkalinity, resulting from sodium carbonate, the surface soil is discoloured and black, and hence the term black alkali is frequently used to designate the non-saline alkali soil.

These two main groups of salt-affected soils differ not only in their chemical characteristics but also in their geographical and geochemical distribution, as well as in their physical and biological properties.

2.3 Chemistry of Sodium

Sodium has been known since times immemorial in the form of salt (NaCl) and soda (Na_2CO_3). Sodium is extremely reactive metal, hence, is not found in a free state. In a combined state, it occurs as sodium chloride (NaCl, rock salt), sodium carbonate (Na_2CO_3), sodium nitrate ($NaNO_3$, chile salt petre), sodium almunate fluoride (Na_3AlF_6, cryolite), borax ($Na_2B_4O_7.10H_2O$), sodium sulphate ($Na_2SO_4O_7.10H_2O$,

glaubers salt) and sodium aluminum silicate ($NaAlS_3O_8$, feldspar). Sodium is a soft silver white metal melts at 97.8°C and boils at 828°C. It is sufficiently malleable, ductile and good conductor of heat and electricity.

2.4 Causes of Salinity

Salt affected land has arisen from natural causes, from the accumulation of salts over long periods of time in arid and semi arid zones (Rengasamy 2002). Weathering of parental rocks releases soluble salts of various types, mainly chlorides of sodium, calcium and magnesium, and to a lesser extent sulfates and carbonates (Szabolcs 1989). Sodium chloride is the most soluble and abundant salt released. The other cause of accumulation is the deposition of oceanic salts carried in wind and rain. Rainwater contains 6-50 mg/kg of sodium chloride and the concentration decreases with distance from the coast. Rain containing 10 mg/kg of sodium chloride would deposit 10 kg/ha of salt for each 100 mm of rainfall per year.

2.5 Why Salinity is a Major Problem?

Substantial areas of the earth's potentially productive lands are affected by soil salinity and alkalinity. The problem of saline soils is increasing because of inadequate irrigation and drainage practices. Further, the agronomic problem of salinity is compounded by the relatively low salt tolerance of many of the major crop plants (Maas and Hoffman 1977). High concentration of soluble salts in the top soil layer is detrimental to profitable agriculture. As mentioned earlier the area of degraded land is increasing with varying degrees of salt accumulations (Legocka and Kluk 2005; Khan *et al.* 2009b; Palma *et al.* 2009; Wei *et al.* 2009)

2.6 Effect of Salinity on Plant Development

2.6.1 Plant Growth

Plant responses to salinity stress have been widely documented in terms of seedling establishment, growth, development and yield with particular reference to characterization of physiological upsets (Greenway and Munns 1980; Lutts *et al.* 1995; Lin and Kao 2001; Al- Ansari 2003). Cruz and Cuartero (1990) have reported that shoot length is one of the most reliable response indicators of salt stress in a wide range of tomato genotypes. Reduced shoot dry weight due to decline in vegetative growth is the most widely used index in studies on salt tolerance in tomato. Prominent growth

reduction, toxic effect and imbalanced nutrition are provoked by NaCl (Alarcon *et al.* 1993; Gersani *et al.* 1993) as well as reduction in biomass due to high concentration of Na^+ and Cl^- (Zhu 2001, Nedjimi and Daoud 2009). *Atriplex prostrata*, a facultative halophyte exhibited significant reductions in plant height and biomass and in the width of the cortex and vascular tissue zones under saline conditions (Wang *et al.* 1997). Roots are the most sensitive organs to salinity stress (Okusanya and Ungar 1984) and play an important role during plant growth and development and are typically the first and critical part of the plant to encounter soil salinity. Sibole *et al.* (2000) reported that salinity induced a decrease in harvest index of bean plants simultaneously with an increase in root index. However, Munns and Termaat (1986) reported that root growth was always less affected than shoot growth under increased salinity. Increasing soil salinity levels have been shown to decrease shoot, root and total dry matter in corn plants (Pessarakli *et al.* 1989; Bar-Tal *et al.* 1991). The relative growth rate was reduced by 25% at 200 mM NaCl, and the total lateral root length decreased as a consequence of inhibition of the lateral root primordia and/or the activation of apical meristems in *Plantago maritima* at 200 mM NaCl (Rubbinigg *et al.* 2004). It has been suggested by Shannon *et al.* (1994) that the overall plant response depends upon the concentrations of salts, the exposure time, and the climatic conditions as well. Plant phenological stages are differentially affected by salinity levels (Manchanda and Garg 2008). Flowering and maturity are delayed due to salinity (Greenway and Munns 1980). Plant genotypes may respond differently to salt stress at different growth stages. Seed germination is the first stage of crop development at which salt stress can be encountered. Intolerable salinity reduces germination, seedling emergence and establishment (Stumpf *et al.* 1986; Ashraf and Foolad 2005). Many species, such as wheat and barley are capable of germinating at very high salt concentrations (over 300 mM NaCl), but the emerging radicle cannot grow further at this level of salinity (Munns and James 2003). Such tolerance among species at germination could be explained by the physicochemical nature of the enlarging process during this developmental stage. For instance, halophytes are no more salt tolerant than glycophytes at the germination stage but quickly establish their superior tolerance through subsequent hypocotyls elongation and seedling development (Malcolm *et al.*

2003). Nevertheless, it has been reported that screening for germination and early seedling development under salt stress conditions can be used to predict salinity response of the mature plant in maize (Maiti *et al.* 1996). In fact, seed germination percentage is a convenient test for screening large numbers of plant genotypes in a rapid manner but must first be correlated to tolerance during emergence, vegetative growth, flowering and maturity if it is to be of value (Maas 1985; Ashraf and Harris 2004). Rice is particularly sensitive to salt stress during the seedling stage with consequent poor crop establishment as well as during reproduction where salinity can severely disrupt grain formation and yield (Moradi and Ismail 2007). They suggested that tolerance to salt at the seedling stage was weakly associated with tolerance during reproduction. The effect of salinity on plant growth is extremely complex and various physiological mechanisms are involved in conferring tolerance to different degree and duration of stress at different plant growth phases (Khan *et al.* 2001; Dashti *et al.* 2009).

The expansion of leaf surface is exponential to unconstrained environment and that a period of stress has been shown to cause first the reduction in the rate of leaf surface addition, followed by a cessation of expansion as the stress intensifies (Terry *et al.* 1983). The reduction in photosynthetic leaf area due to increased soil salinity levels has been shown in various plant species (Munns 2002; Jimenez *et al.* 2003). However, the reduction in the total leaf area was found dependent on the level of soil salinity, plant phenological stages and the experimental crop species (Syeed 2008). Although, low salinity doses had no significant effect on leaf area (Kulkarni and Karadge 1991), but high concentrations of NaCl caused great reduction in the leaf area of sugar beet cultivars (Ghoulam *et al.* 2002). The leaf area was reduced by 60% in *Erythrina variegata* treated with 250 mM NaCl (Muthuchellian *et al.* 1996). In *Atriplex prostrata*, a facultative halophyte, length of internodes and leaf area significantly decreased with increased salinity (Wang *et al.* 1997).

2.6.2 Photosynthetic Traits

Photosynthesis is the engine driving plant growth and development on the globe which in fact is the light-driven series of reactions that plants use to convert carbon dioxide into carbohydrates (Salisbury and Ross 1992; Taiz and Zeiger 2002).

Photosynthesis and its related physiological variables are invariably affected by the soil salinity levels in various plant species (Ziska *et al.* 1990; Nieves *et al.* 1991; Munns 1993; Parida and Das 2005; Munns *et al.* 2006; Chaves *et al.* 2009). The available literature suggests that the reduction in photosynthesis under saline conditions may be due to (i) reduced conditions of CO_2 intake by the photosynthetic organs (ii) modification in the structure and function of photosynthetic organelles (iii) alterations in light reactions and/or (iv) reduced rate of transport of assimilates and intermediary compounds to the metabolic sinks (Manchanda and Garg 2008; Chaves *et al.* 2009). In addition, salinity induced reduction in photosynthesis has been attributed to salinity caused changes in nutrients and ions (Downton 1977; Papp *et al.* 1983; Yeo *et al.* 1985; Rawson 1986). Salinity caused reduction in photosynthetic pigments and stomatal conductance directly and/or indirectly affects photosynthesis (Flexas *et al.* 2004, 2007). A significant reduction in photosynthesis, stomatal conductance, leaf water potential, and chlorophyll pigments was observed under high salinity stress in *Pueraria lobata* (Al-Hamdani 2004). In some *Medicago sativa* genotypes, reduction in photosynthesis at high salinity level was primarily due to reduction in stomatal conductance. A concomitant decline in the stomatal conductance indicated that exchange of gases was disturbed leading to a reduction in the CO_2 uptake and hence a decline in the photosynthetic rate (Anand *et al.* 2000). It has been shown that salinity-induced reductions in photosynthesis resulted from decreased CO_2 availability (Flexas *et al.* 2004, 2007) or in the alterations of photosynthetic metabolism (Lawlor and Cornic 2002) or from secondary effects such as oxidative stress.

2.6.3 Ion Accumulation and Toxicity

Salinity is a ubiquitous problem of the world, contributing several affects on plant growth and development including ion accumulation and their transportation from root to shoots (Kwon *et al.* 2009). It has been suggested that, in salt stress, the rate of increase in ambient salt concentrations can lead to ion toxicity (primarily due to the accumulation of Cl^- and Na^+), which leads to decrease in chemical activity causing cells to lose turgor and simultaneously to alterations in various physiological processes (Serrano *et al.* 1999; Manchanda and Garg 2008). In fact, excess Na^+ and Cl^- causes negative impacts on the acquisition and homeostasis of essential nutrients (Greenway

and Munns 1980; Grattan and Grieve 1992) and causes conformational changes in protein structure and membrane depolarization (Manchanda and Garg 2008). It is suggested that Cl^- ion accumulation adversely affects photosynthesis (Khayyat *et al.* 2009) Accumulation of Na^+ and/or Cl^- in leaves of various plant species treated with a range of salt concentrations has been widely investigated and reviewed (Grattan and Grieve 1992; Netondo *et al.* 2004; Munns 2005; Manchanda and Garg 2008). Hernandez and Almansa (2002) examined the effect of short-term salt stress and recovery in pea and observed a linear increase in Na^+ concentration, whereas in recovered plants, they observed a slight reduction in leaf Na^+ concentration. There exists a differential pattern of accumulation and/or partitioning of Na^+ and Cl^- in plants (Zhao *et al.* 2002) and affects root growth and leaf area (Maggio *et al.* 2007). Further, Maggio *et al.* (2007) suggested that the sharp increase in leaf Cl^- at higher root-to-shoot ratio is indicative of the accumulation of Cl^- which was mainly associated to the increased root biomass and a reduced leaf area rather than an increased transpiration flux, since they found decreases in the latter with salinity. These results highlight the importance of the root-shoot ratio in modulating ion accumulation and possibly salt stress adaptation in plants (Moya *et al.* 1999; Estan *et al.* 2005; Maggio *et al.* 2007).

Genotypic variation in the accumulation and partitioning of Na^+ and Cl^- in salinity treated plants has also been reported by several workers (Brugnoli and Lauteri 1991; Abd-Alla *et al.* 1998; Saadallah *et al.* 2001; Serraj *et al.* 2001; Jungklang *et al.* 2003; Malencic *et al.* 2003; Sibole *et al.* 2003; Garg and Singla 2004; Verdoy *et al.* 2004; Parida and Das 2005; Zhao *et al.* 2007). Na^+ was the major cation that accumulated in roots and stems of sorghum (Netondo *et al.* 2004) and *Olea europeae* (Ahmad *et al.* 2008) as salinity increased. In fact, Na^+ and/or Cl^- preferentially accumulate in roots over shoots as a mechanism of salt tolerance (salt exclusion) and maintain a substantial potential for osmotic water uptake into the roots and restricts the spread of Na^+ to shoots (Renault *et al.* 2001; Chartzoulakis *et al.* 2002; Loreto *et al.* 2003; Viegas *et al.* 2003; Netondo *et al.* 2004; Chartzoulakis 2005; Aydi *et al.* 2008). However, Na^+ may also be accumulated more in shoots than roots. Tester and Davenport (2003) showed that some halophytes survived by accumulating up to 50% of their dry weight as Na^+ in shoots. The same authors suggested that mechanisms

minimizing damages due to Na^+ accumulation must operate in a coordinated manner. Results obtained by Yamamauchi *et al.* (1997) in common bean showed particularly that: (i) growth is negatively correlated with leaf Na^+ and Cl^- concentrations and positively with the amounts of these elements accumulated in roots, (ii) for each element, the correlation coefficient relative to Na^+ are higher than those relative to Cl^- (iii) leaf growth depends on the aptitude of the cultivars to retain Na^+ in roots. In the same way, soybean, the salt tolerant variety (Lee) maintained its foliar Cl^- contents at low level, as compared to the sensitive variety (Jackson). da Silva *et al.* (2008) observed in young *Spondias tuberosa* plants that Na^+ content in leaves increased linearly with increase in NaCl levels, reaching the highest value in plants subjected to 100 mM NaCl.

Maggio *et al.* (2007) reported in salt-treated *Lycopersicon esculentum* plants that older leaves accumulated more Na^+ than younger leaves. It has been previously established that once the Na^+ enters into the root cells it is extruded from the cytoplasm into the apoplastic space (Shi *et al.* 2000, 2003) and/or compartmentalized into the vacuole (Apse *et al.* 1999; Zhang and Blumwald 2001). At low salinity, Na^+ is mostly removed from the cytoplasm through the above-mentioned mechanisms. The remaining Na^+ (the amount that is not stored into the vacuole) follow the transpiration water flux. Consequently, a higher Na^+ accumulation is observed in leaves that are transpiring since longer time (mature leaves) (Maggio *et al.* 2007).

2.6.4 Water Relations

High soluble salts of soil-solutions generate a low osmotic potential that lowers the soil-water potential which in turn, cause ion imbalance of the cellular ions, ion toxicity and osmotic stress and perturbs the general water balance of plants (Khan *et al.* 2002; Khan and Panda 2002; Panda and Khan 2003; Demiral and Turkan 2005; Mandhania *et al.* 2006). In fact, the stress caused by ion concentrations allows water gradient to decrease, making it more difficult for water and nutrients to move through the root membrane (Volkmar *et al.* 1998). In turn, the water uptake slows, and as the osmotic effect spreads from the root membrane to the internal membranes, the ion concentration inside the plant alters the solute balances (Volkmar *et al.* 1998). Once high concentrations of salt have reached inside the plant, the development of tissue and

organs is altered. The salt causes a slower rate or shorter duration of expansion of cells and this compromises the size of the leaves (Volkmar *et al.* 1998). Water and osmotic potential of plant have been found more negative with an increase in salinity levels (Khan 2001; Meloni *et al.* 2001; Romeroaranda *et al.* 2001). Relative water content, leaf water potential (Ψ_w), water uptake, transpiration rate, water retention and water-use efficiency were found decreased under short-term salinity in jute (Chaudhuri and Chaudhuri 1997). Aziz and Khan (2001) noticed an increase in leaf water potential and osmotic potential in *R. mucoronata* with increase in media salinity. Salinity-induced reductions in root and leaf water potential have been reported by several workers (Sayed and Gadallah 2002; Maggio *et al.* 2007; Ashraf *et al.* 2008). Maggio *et al.* (2007) observed decrease in total Ψ_w at increasing EC of the nutrient solution in tomato. Ψ_w was -0.70 MPa in the control plants whereas it reached -1.21 MPa at the highest salinity (15 dS/m). Salinity reduces water potential in *Psidium guajava* (Távora *et al.* 2001), avocado (Chartzoulakis *et al.* 2002), mangabeira tree (Albuquerque 2004), and sugar apple (Nogueira *et al.* 2004).

2.6.5 Oxidative Stress

A secondary effect of salinity stress is increase in the production of ROS, which include singlet oxygen, superoxide anion radicals, hydroxyl radicals, and H_2O_2 (Smirnoff 1998; Apel and Hirt 2004). In fact, salinity stress causes reduced uptake of CO_2 as a result of stomatal closure (Khan and Panda 2008). Limited CO_2 fixation due to stress conditions leads to a decrease in carbon reduction by the Calvin cycle and decrease in oxidized $NADP^+$ to serve as an electron acceptor in photosynthesis. When ferredoxin is over reduced during photosynthetic electron transfer, electrons may be transferred from photosystem I (PSI) to oxygen to form superoxide radicals by the process called Mehler reaction, which triggers chain reactions that generate more aggressive ROS including $O_2^{\cdot-}$, $^{\cdot}OH$, and H_2O_2 (Baisak *et al.* 1994; Polle and Rennenberg 1994; Iturbe-Ormaetxe *et al.* 1998; Lawlor 2002) which ultimately causes oxidative stress in plants (Asada 1994; Baisak *et al.* 1994; Mittler 2002; Hsu and Kao 2003). Salt stress accrued oxidative stress in plants has been investigated and reviewed extensively (Bray *et al.* 2000; Zhu 2000; El-Tayeb 2005; Azevedo Neto *et al.* 2004; 2006; 2008; Sekmen *et al.* 2007; Khan and Panda 2008; Turkan and Demiral 2008,

Turhan *et al.* 2008). The presence of high concentration of ROS causes oxidative damage to photosynthetic pigments, bio-molecules such as lipids, proteins and nucleic acids, leakage of electrolytes via lipid peroxidation, which results in the disruption of the cellular metabolism (Asada 1994; Reddy *et al.* 2004; Mobin and Khan 2007).

2.6.6 Lipid Peroxidation

Oxidative stress is generally estimated in terms of lipid peroxidation and membrane damage [Thiobarbituric acid reactive substances (TBARS)] and H_2O_2 contents (Sudhakar *et al.* 2001; Mittler 2002; El-Tayeb 2005; Polesskaya *et al.* 2006; Sekmen *et al.* 2007; He and Zhu 2008; Khan and Panda 2008). Under saline environments, the plant lipid metabolism is interrupted as a result of oxidative damage to membrane lipids by ROS and lipid peroxidation (Hernandez *et al.* 2002). Bor *et al.* (2003) observed NaCl dose-dependent increase in the content of TBARS in leaves of sugar beet (*Beta vulgaris*) and *B. maritime*. Sekmen *et al.* (2007) reported a differential response of two cultivars of *Plantago* differing in salt tolerance exposed to 0, 100 and 200 mM NaCl. The level of MDA in the leaves increased under salt stress in *P. media* but showed no change and decreased in *P. maritima* at 100 and 200 mM NaCl, respectively. Koca *et al.* (2006) reported increase in lipid peroxidation in terms of increase in the content of MDA in salt (0, 70 and 140 mM NaCl) treated *Lycopersicon esculentum* and *L. pennellii* sampled after 0, 6, 12 and 84 days of salt treatment initiation. Lipid peroxidation in leaves of *L. pennellii* was almost the same throughout the experimental period. However, MDA content in leaves of *L. esculentum* showed a significant age-dependent increase on day 84 under control conditions. MDA contents in this species were always higher than in *L. pennellii* under both salt treatments. A lower level of lipid peroxidation, hence a lower degree of membrane damage in *L. pennellii* than in *L. esculentum* leaves might be resulted from the higher activities of enzymatic antioxidants in *L. pennellii*. Sairam *et al.* (2005) reported genotypic variation in the response of *Triticum aestivum* genotypes in terms of accumulation of TBARS in leaves under 100 and 200 mM NaCl. Leaf TBARS content increased with salinity in all *T. aestivum* genotypes (HD2009, HD2687, KRL19 and Kharchia65). However, TBARS content was highest in HD2687 in both control and salt stressed plants and minimum in Kharchia65, while HD2009 and KRL19 showed intermediate response. Khan and

Panda (2008) studied the salinity-induced lipid peroxidation in terms of MDA accumulation in roots of two *Oryza sativa* cultivars treated with 50, 100 and 150 mmol/l NaCl. MDA level increased and the ratio was higher in *O. sativa* cv. Begunbitchi compared to Lunishree.

The accumulation of H_2O_2 in plants under stress conditions is also an indicator of oxidative stress. It is an endogenous signaling molecule involved in plant responses to abiotic and biotic stresses including salinity. There are reports that salt treatment increases the content of H_2O_2 thus disrupting its permeability or induce oxidative stress in plant tissues (Hernandez *et al.* 2000; Jain *et al.* 2001; Demiral and Turkan 2005; Mandhania *et al.* 2006). Sairam *et al.* (2005) investigated the relevance of H_2O_2 accumulation in tolerance of *T. aestivum* genotypes grown under 100 and 200 mM NaCl. H_2O_2 content increased with increasing NaCl concentrations in all the genotypes (HD2009, HD2687, KRL19 and Kharchia65). Cultivar HD 2687 followed by HD2009 showed higher H_2O_2 content in control and stressed plants than other genotypes. He and Zhu (2008) reported a sharp increase in H_2O_2 content in leaves of *L. esculentum* treated with 100 mM NaCl. Fadzilla *et al.* (1997) reported severe oxidative damage with elevated concentrations of H_2O_2 in *O. sativa* plants exposed to salt stress.

2.6.7 Modulation of Key Antioxidant Enzymes

To minimize the effects of oxidative stress, plant cells have evolved a complex antioxidant system, which is composed of low-molecular mass antioxidants (glutathione, ascorbate, carotenoids) as well as ROS-scavenging enzymes, such as SOD, CAT, APX, guaiacol peroxidase (GPOX, EC 1.11.1.7) and GR (Alscher *et al.* 1997; Apel and Hirt 2004; Heidari and Mesri 2008, Antioxidants are related to various abiotic stresses including salinity (Khosravinejad *et al.* 2008; Athar *et al.* 2009; Gama *et al.* 2009). SOD reacts with the $O_2^{\cdot}$ to produce H_2O_2. Hydrogen peroxide is scavenged by CAT and peroxidases (POX, EC 1.11.1.7). Among peroxidases, APX and glutathione peroxidase (GPX, EC 1.11.1.9) use AsA and GSH as electron donors, respectively, are well known for their role in H_2O_2 detoxification in plants. GR is responsible for the reduction of GSSG for the chain reactions of scavenging H_2O_2 by APX and GPX to be completed and continued (Mittler 2002; Apel and Hirt 2004).

A large body of evidence has shown that the antioxidant enzyme systems are altered under abiotic stresses, including salinity. The quantitative and qualitative aspects of changes are often related to the levels of resistance to salinity. The loss of the ability to scavenge free radicals during stress is generally attributed to a decrease in the activity of antioxidant enzymes such as SOD, APX and GR (Pastori and Trippi 1993; Mittova *et al.* 2002; Desingh and Kanagraj 2007). In *O. sativa*, the salt-tolerant varieties have higher SOD activity and lower lipid peroxidation than the salt-sensitive varieties (Dionisio-Sese and Tobita 1998). In *L. esculentum* and citrus, salt-tolerance is attributed to the increased activities of SOD, APX, and CAT (Gueta-Dahan *et al.* 1997; Mittova *et al.* 2004). Further supporting evidence on the involvement of antioxidant enzymes in salt tolerance has been provided by transgenic plants with a reduced or an increased expression of antioxidant enzymes. The antisense plants with reduced CAT activity are hypersensitive to salt and other oxidative stresses (Willekens *et al.* 1997). Increased protection to salt stress has been demonstrated by the overexpression of cytosolic APX (Torsethaugen *et al.* 1997). Enhanced oxidative stress tolerance was also observed in the plants overexpressing Fe-SOD (Van Camp *et al.* 1996). Kim *et al.* (2005) studied the responses of antioxidant enzymes in shoot and root of *Hordeum vulgare* plants exposed to 200 mM NaCl for 1, 2 and 5 days. In general, the activities of antioxidant enzymes were increased in the root and shoot under saline stress. But the increase was more significant and consistent in the root. The activities of SOD, CAT, APX, POX, and GR were increased significantly in the root within 1 day after treatment, and various elevated levels were maintained by 5 days after treatment. Among antioxidant enzymes, CAT activity was increased the most drastically. The significant increase in the activities of SOD, CAT, APX, POX, and GR in the NaCl-stressed *H. vulgare* root was highly correlated with the increased expression of the constitutive isoforms as well as the induced ones. In addition, the H_2O_2 content in the root was most highly correlated with the CAT activity, indicating the role of increased CAT in H_2O_2 detoxification under salinity stress. Telesinki *et al.* (2008) reported a gradual increase in CAT and POX activities in *Phaseolus vulgaris* plants treated with 10, 30, 50 mM/kg NaCl in soil. Salinity stress-caused increase in POX activity has been reported in *Morus alba* (Sudhakar *et al.* 2001), *Glycine max* (Ghorbanli *et al.* 2004), *L.*

esculentum (Rahnama and Ebrahimzadeh 2005) and *Phaseolus vulgaris* (Telesinki *et al.* 2008). The activity of antioxidant enzymes has been reported to increase under saline conditions in the case of salt tolerant *Gossypium hirsutum* (Meloni *et al.* 2003), shoot cultures of *Oryza sativum* (Fadzilla *et al.* 1997), *Cucumis sativus* (Lechno *et al.* 1997), *Triticum aestivum* shoot (Menogeuzzo *et al.* 1999) and *Pisum sativum* (Hernandez *et al.* 1999). Desingh and Kangaraj (2007) studied important antioxidant enzymes (SOD, APX and GR) in two *Gossypium hirsutum* varieties (Arya-Anubam and LRA-5166) treated with 0 mM (control), 50 mM, 100 mM, and 150 mM NaCl. They observed a progressive increase in both *G. hirsutum* varieties at all salt concentrations applied. However, the activities of all enzymes were markedly higher in var. Arya-Anubam than in var. LRA-5166 at all salinity levels. Variety Arya-Anubam showed a significantly increased activity of SOD (175.93 Units/mg protein/min), APX (75.32 mmol/mg protein/min) and GR (99.47 µmol/mg protein/min) at 150 mM salt concentration when compared to control plants (124.63 Units/mg protein/min, 30.65 mmol/mg protein/min, 52.19 µmol/mg protein/min, respectively).

2.6.8 Yield and Its Attributes

The detrimental effects of high salinity on plants can be observed at the whole plant level, such as a significant reduction in plant growth, decrease in productivity, and even the death of plants. The accumulation of Na^+ in leaf tissues usually results in the damage of old leaves, which shortens the lifetime of individual leaves, thus reducing the net productivity and crop yield (Munns 1993, 2002). Increased NaCl levels result in a significant decrease in root, shoot, and leaf biomass and an increase in root/shoot ratio in cotton (Meloni *et al.* 2001). In addition, salt stress can also induce or accelerate senescence of the reproductive organs. Saqib *et al.* (2008) studied the effect of intensification of sodicity on salinity-induced effect of on grain yield and yield component in *T. aestivum* cultivars (Inqalab-91; Aqaab and MH-97). Salinity (EC 15 dS/m) and salinity × sodicity (EC 15 dS/m and SAR 35) significantly reduced the grain yield of all the three *Triticum aestivum* genotypes. The reduction in the grain yield due to salinity × sodicity was also found significantly greater than due to salinity alone except for the genotype Inqlab-91. The genotype MH-97 produced the minimum grain yield in salinity as well as salinity × sodicity treatments, whereas Inqlab-91 and Aqaab

were non-significant. The 100-grain weight of Aqaab and Inqlab-91 was not reduced significantly by salinity. The 100-grain weight of Inqlab-91 was the highest in salinity and salinity × sodicity treatments with non-significant differences between the treatments, whereas the 100-grain weight of MH-97 was the lowest in both the treatments. The spike length and straw weight of all the genotypes were reduced by salinity as well as salinity × sodicity, but the number of spike bearing tillers per plant of Inqlab-91 was not reduced significantly by salinity × sodicity. The impact of salinity on yield has been found to be dependent on the timing, speed of onset, intensity and duration of the stress. The exposures of crop plants during vegetative and/or reproductive phases have been shown to significantly affect the crop productivity (Burman *et al.* 2003). Salinity reduces the yield of rice approximately by 45%, which mainly results from spikelet sterility and reduced seed weight (Asch and Wopereis 2001). In field-grown *Gossypium hirsutum*, salt stress was a major reason for seed abortion, leading to both yield loss and bad fiber quality (Davidonis *et al.* 2000). Nearly 90% of the ovules of *Arabidopsis* aborted and smaller fruits resulted when roots were incubated for 12 h in a hydroponic medium supplemented with 200 mM NaCl (Sun *et al.* 2004). Kumar *et al.* (2006) studied the effect of salinity on oil yield, seed yield and its components in *Brassica juncea* cultivars (RH8113, salt sensitive; CS52, salt tolerant). Salt stress invariably reduced the number of siliqua per plant in both cultivars. At 150 mM NaCl, percent reduction in number of siliqua/plant was found higher in RH8113 (43%) than in CS52 (31%). Similarly, salinity-induced reduction in seed yield was noticed higher in RH8113 than CS52. Oil content in both cultivars declined significantly as a function of salt stress.

2.7Salinity-Stress Mitigation Strategies

Salinity and drought are the major limiting factors in agricultural productivity (Boyer 1982; Bartels and Sunkar 2005), and they are becoming more serious problems with global warming (Gale 2002; Grover *et al.* 2003). Furthermore, although salinity in soil could be somewhat relieved through farm management practices, such as better irrigation practice, phase farming, intercropping and precision farming (Munns 2002), it takes a long time and high cost to improve soil quality suitable for crop growth. Therefore, it is imperative that researchers develop breeding strategies and technologies

to make crops more productive under stressful environments (Cushman and Bohnert 2000).

Concerted efforts in conventional breeding programmes have led to the development of a number of improved cultivars, but the yield levels have been virtually static owing to susceptibility of the cultivars to salinity stress, and/or a limited genetic variability in the cultivar germplasm. The subsequent steps and methods adopted till date for improving salt stress can be summarized as follows: (i) Screening of germplasm-intercultivar variation, (ii) Screening of variable material of a single cultivar-intervarietal variation, (iii) Intergeneric hybridization, Conventional breeding programmes, (iv) Induced mutations, (v) Use of in vitro selection, (vi) Pooling of physiological traits, (vii) Use of marker-aided selection, (viii) Transgenics, (ix) Use of osmoprotectants such as proline, soluble sugars, polyols, glycinebetains, (x) Use of antioxidants and growth regulators and (xi) Use of mineral nutrient management strategies.

2.7.1 Mineral Nutrient Management – A Sustainable way for Improving Plant Growth, Productivity and Stress tolerance

No one knows when and where the practice of burying a fish beneath the spot where a few seeds of corn were to be planted originated, but it was common among North American Indians when Columbus discovered America and is evident that the value of fertilizers was known to the primitive peoples. Farm manures were in common use by the Romans and have utilized almost from the time, animals were first domesticated and crops grown. But the origin of plant nutrition as a science can be traced as far back as Aristotle. However, the modern scientific study of the effect of mineral nutrients, and the usefulness of chemical fertilizers can be ascribed to the work of Boussingoult, Liebig and Laws and Gilbert (Russell 1950). By the early twentieth century 10 elements had been identified as essential for proper nutrition. These were carbon, hydrogen and oxygen which are supplied by atmosphere; and nitrogen, potassium, phosphorus, sulfur, calcium and iron supplied by the soil. The first 40 years of twentieth century witnessed the addition of manganese, boron, copper, zinc, molybdenum and chlorine to the list of essential mineral nutrients, which very recently nickel has been added to this list (Marschner 1995).

Keeping in view the specific nature of the problem of the thesis the following pages deal with sulfur and its effect on crops with special emphasis on mustard.

2.7.1.1 Sulfur

Sulfur is one of the 17 essential elements for plants growth. Sulfur is essential nutrient, both for plants and animals. It is fourth in importance after N, P and K, considered vital for proper plant growth and development (Syers *et al.* 1987). The importance of S as a plant nutrient has been recognized for a long time but active research started in the second half of the 20th century when widespread S deficiencies were observed (Duke and Reisenauer 1986). Morris (1988) stated that "Plant nutrient sulfur" (PNS) was required by plants in amount similar to P and is important to the plant for protein formation and other functions. Sulfur has variety of vital functions within the plant. In fact, inorganic sulfur is converted to nutritionally and functionally important S-containing compounds like cysteine, methionine, several co-enzymes, thioredoxins, sulpholipids and vitamins i.e. biotin, thiamine and ferredoxin through a cascade of enzymatic steps (Hell 1997; Saito 2000). Sulfur is important in the formation of sulfhydryl (S-H) and disulphide bonds (S-S). A good part of the sulfur incorporated into organic molecules in plants is located in thiol (-SH) groups in proteins (cysteine-residues) or non-protein thiols. Due to their particular redox properties, thiol groups can be oxidized forming disulphides (S-S groups). These bonds are important for stabilization of protein structures and in many enzymes thiol groups form the active centers (Noji and Saito 2003). As a part of the cysteine-molecule, the sulfur group, called a thiol, is strongly nucleophilic, making it ideally suited for biological redox processes. Redox control regulates enzymes and protects against oxidative damage.

2.7.1.1.1 Forms of Sulfur in Soil

Sulfur is continuously cycled between inorganic and organic forms. Three broad fractions of organic S have been identified: (i) Ester sulfate (ii) C-bonded S (mainly amino acids) and (iii) Residual S (Tabatabai 1982). These transformations in soils are brought about primarily by the action of microbes, although chemical processes such as oxidation of iron sulfide have also been reported. The major microbial processes which

are thought to be involved in transformations are mineralization, immobilization, oxidation and reduction. They are discussed briefly below:

2.7.1.1.1.1 Mineralization

The mineralization of S in soil refers to the break down of large organic S molecules in soil to smaller units and finally to inorganic sulphate, despite many reports, the mechanism of S-mineralization in soil are still not known completely (Haque and Walmsley 1972). Carbon stabilized elements are mineralized as a result of carbon-oxidation to provide energy, as is the case with carbon-bonded organic S-compounds in soils in contrast to elements in ester forms (Saggar *et al.* 1981).

2.7.1.1.1.2 Immobilization

This is a process by which micro organisms convert simple inorganic S-molecules to organic compounds and eventual incorporation into humus. Under conditions when organic matter is rapidly accumulating considerable amount of sulphate-S may be transformed to organic forms.

2.7.1.1.1.3 Oxidation

The S-oxidizing organisms are autotrophic, ubiquitous and capable of very rapid oxidation rate in vitro. They belong to the family thiobacteriaceae, hydrogen sulfide, elemental S, thiosulphate and polythionates are oxidized to sulphate by various members of the thiobacilli.

The dissimilatory sulphate reduction in soil is brought about by obligate anaerobes belonging to S-reducing bacteria, *Desulphotomacium* and *Desulpho-vibrio*. These bacteria use sulphate as the terminal electron acceptor in their respiratory processes (Roy and Trudinger 1970). These all S-reducing bacteria contain cytochrome, the respiratory pigments, which act as electron donor in the reducing process.

Sulphate S	Dissolution
Elemental S	Oxidation
Polysulphides (SX)	Decomposition Elemental S Oxidation
Thiosulphate	Decomposition Elemental S Oxidation
Organic S	Mineralization

2.7.2 Sulfur Cycling

Sulfur cycling has important implication because cyclic S is a source of S for crops. The most obvious illustration of S cycling is the soil-plant-rain pathway. The level of S-accruing to soils and crops via wet deposition approaches approximately 0.1 to 0.2 mg/l. Another pathway is the atmosphere-plant-soil route. This is called as dry deposition and is important in industrial and residential areas where fossil fuels are burned.

2.7.3 Reasons for Sulfur Deficiency

Sulfur deficiencies are being reported in ever increasing numbers in crops through out the world (Tisdale *et al.* 1986; Morris 1987; Messik *et al.* 1992). Morris (1987, 1988) lists the changes as follows:

1. Replacement of fertilizers containing S, such as ammonium sulphate and single supper phosphate, applied normally to supply N and P respectively, with higher analysis fertilizers such as urea, and triple super phosphate which contain little or no S.
2. Use of crop residues for food or fuel in developing countries (Bhuyian 1992).
3. Declining reserves of soil S.
4. Greater control of industrialized emissions of S and decreased use of high S fuels such as coal.
5. Decreased use of pesticides which contain S.

Not all the factors apply in all countries; in Indian context, decreased incidental addition of S is a probable reason for its deficiency in the crops (Pasricha and Aulakh 1991). A study was conducted by Tiwari (1994) to access the pattern of deficiency in the soils of western Uttar Pradesh (India). The S-deficiency was most extensive in soil of the district of Hardori (59%) followed by Aligarh (41%), Agra (37%), Unnao (32%), Kanpur (31%), Mainpuri (24%), Hamirpur (24%), Farukhabad (19%), Jalaun (18%) and Lalitpur (17%).

2.7.4 Sulfur Assimilation and Importance in Plant Development

Plants are the major food source for humans and other animals, providing carbohydrate, protein, lipids and vitamins (Imsande 2003). Importance of S as a plant nutrient is becoming more imminent due to its effect on crop productivity and quality.

Agronomic responses of crop growth and yield to the addition of S-fertilizer are well documented. Several greenhouse and field experiments with various crop plants as test plants have indicated that effect of S is primarily on the number of grains per pod per siliqua or spike indicating that S-deficiency either increases the mortality of flowers per florets or reduces the initiation of florets (Archer 1974; Islam *et al.* 1999; Ahmad *et al.* 2005). Other yield components such as number of pods per siliqua and tillers and 100(0)-grain weight are affected to a lesser extent by S-availability. A large number of studies have reported a marked influence of applied S on the yields of several cereals, pulses, oilseeds, vegetables, forages and other crops (Pasricha *et al.* 1987; Tandon 1991; Ahmad *et al.* 1998; Aulakh and Pasricha 1998). Oilseed rape has a high requirement for S and is particularly sensitive to any shortfall in S supply (Ahmad *et al.* 2005). Yield responses of oilseed rape to S supply have been reported in many countries (Walker and Booth 1992). In fact, S is an important nutrient for oilseed rape due to its association with yield and also a range of quality factors. It is required by *Brassicas* for the synthesis of the S-bearing compounds glucosinolates. Seeds from many plants, especially of legume crop, contain low concentrations of small 2S proteins that are relatively rich in cysteine and methionine (Shewry and Pandya 1999). *Glycine max* also produces low molecular weight polypeptides that contain disproportionately high methionine content (George and de Lumen 1991; Paek *et al.* 2000). In fact, the availability of reduced S (i.e., cysteine and methionine) is the rate-limiting factor for the regulation of β-conglycinin chains that usually synthesized only during late seed development (Meinke *et al.* 1981).

2.7.4.1 Enzymes of Sulfur Assimilation

Sulfur is found in soil in the form of sulfate and through a set of reaction is converted to sulfide and into an N/C-skeleton forms cysteine or its homologues (Droux 2004). The assimilation of sulfate could be summarized in four steps: (1) Uptake of sulfate (2) Activation of sulfate (3) Reduction of sulfate (4) Synthesis of cysteine. Sulfate uptake is facilitated by sulfate transporters; once sulfate is within cells, it can be stored or can enter the metabolic stream. Metabolism of sulfate is initiated by its activation by the reaction of adenylation catalyzed by ATP-sulfurylase. The reaction product adenosine 5′-phosphosulfate (APS) is a branch point intermediate, which can

be channeled toward reduction or sulfation (Leustek *et al.* 2000). Activation of sulfate reduction is the dominant route for assimilation and is carried out in plastids (Brunold and Suter 1989; Rotte 1998; Leustek *et al.* 2000; Saito 2000). APS is reduced to sulfite by APS-reductase (APR) (Leustek and Saito 1999; Kopriva and Koprivova 2003), finally sulfite is reduced to sulfide by sulfite reductase (SiR). Sulfide is then transferred to activated serine by O-acetylserine(thiol)lyase (OAS-TL) to form cysteine. The formation of cysteine is a direct coupling step between S and N assimilation in plants (Brunold 1990; 1993; Brunold *et al.* 2003). Cysteine is the precursor or S-donor for most other organic S-compounds in plants. In addition, cysteine is the precursor of GSH, a low molecular weight, water soluble non-protein thiol compound which functions in protection of plants against varied environmental stresses (De Kok *et al.* 2005).

2.7.4.1.1 ATP-sulfurylase

Activation of the relatively inert sulfate occurs through binding to ATP catalyzed by ATP-sulfurylase. In fact, ATP-sulfurylase catalyzes the first step in sulfate assimilation, the adenylation of sulfate to APS from ATP and sulfate.

The formation of APS is an energetically unfavourable process, which is driven forwards by the consumption of APS by subsequent reactions, reduction to sulphite by APS-reductase or phosphorylation to PAPS by APS-kinase. Because of the thermodynamic balance, ATP-sulfurylase enzyme activity is routinely measured either in the back reaction by measurement of ATP synthesized from APS and pyrophosphate or indirectly by the molybdolysis assay which determines the rate of AMP production (Segel *et al.* 1987). *Arabidopsis thaliana* contains a three-member, highly homologous, expressed gene family encoding plastid localized forms of ATP-sulfurylase; APS1, APS2, and APS3 (Murillo and Leustek 1995). All three cDNA clones functionally complement a met3 (ATP-sulfurylase) mutant strain of *Saccharomyces cerevisiae* (Murillo and Leustek 1995). APS1 is the most highly expressed member of this gene family. The APS polypeptides share homology with ATP-sulfurylase from fungi, a marine worm and a chemoautotrophic bacterium, but not from *Escherichia coli* or *Rhizobium meliloti* (Murillo and Leustek 1995). Analysis of recombinant APS3 indicates that the protein is structurally and kinetically similar to fungal ATP-

sulfurylase, but very different from the E. coli enzyme. The APS3 polypeptide is a homotetramer. Despite the sequence, structural, and kinetic differences between higher plant and *E. coli* ATP-sulfurylases, APS2 and APS3 are able to functionally complement to *E. coli* cysD and cysN (ATP-sulfurylase) mutant strains (Murillo and Leustek 1995). Plant ATP-sulfurylase is a homotetramer of 52-54 kDa polypeptides (Murillo and Leustek 1995). In plants, ATP-sulfurylase activity was detected in chloroplasts and in the cytosol of *Spinacea oleracea* leaves (Lunn *et al.* 1990; Renosto *et al.* 1993) and in proplastids of *Pisum sativum* roots (Brunold and Suter 1989). However, ATP-sulfurylase was found in the cytosol and mitochondria in *Euglena gracilis* (Li *et al.* 1991). Low ATP-sulfurylase activity was measured in etiolated *P. sativum* seedlings, which increased after transfer into the light, but decreased again in the leaves during further incubation (von Arb and Brunold 1986). Klonus *et al.* (1994) reported an ATP-sulfurylase mRNA in *Solanum tuberosum* leaves, stems, and roots, but not in tubers. There are reports of both decreasing and increasing trend of ATP-sulfurylase activity in various plants. In poplars, (*Populas tremulax*, *P. alba*) ATP-sulfurylase activity diminished slowly with the leaf age (Hartmann *et al.* 2000). In *Arabidopsis*, the foliar ATP-sulfurylase activity continually declined during the plant growth. During this time the more abundant chloroplastic ATP-sulfurylase activity was found decreasing, while cytosolic activity was increased (Rotte and Leustek 2000). This observation indicates different functions of ATP-sulfurylase in the two compartments, sulfate reduction in the plastids and activation of sulfate for synthesis of sulphonated compounds in the cytosol (Rotte and Leustek 2000). ATP-sulfurylase activity may be localized to bundle sheath cells of chloroplasts. There are reports that approximately 80-100% of total leaf ATP-sulfurylase activity in *Zea mays* may be confined to bundle sheath cells (Gerwick *et al.* 1980; Passera and Ghisi 1982; Schmutz and Brunold 1984). Furthermore, it was revealed that mRNA of ATP-sulfurylase is present exclusively in RNA isolated from bundle-sheath cells of *Z. mays*, revealing that in *Z. mays*, the intercellular distribution of ATP-sulfurylase is regulated on the transcriptional level (Kopriva *et al.* 2001).

2.7.4.1.2 APS-reductase

Sulfate reduction is the dominant route for assimilation and is carried out in plastids (Brunold and Suter 1989; Rotte 1998). In fact, the sulfate reduction is carried out in two steps i.e., (a) APS-reductase transfers two electrons to APS to produce sulfite, and (b) Sulfite reductase transfers 6 electrons from ferredoxin to produce sulfide.

APS-sulfotransferase, 5′-adenylylphosphosulfate reductase, adenosine 5′-phosphosulfate reductase are synonyms of APS-reductase (Setya *et al.* 1996; Suter *et al.* 2000). APS-reductase reduces the sulfate residue of APS into sulfite. APS-reductase is a unique enzyme. It is now known that APS-reductase possesses a transit peptide that allows translocation of the mature protein to plastids. Kopriva and Koprivova (2004) observed in vivo study that APS-reductase is present as a homodimer most probably linked by a disulfide bond of the conserved cysteine residue. Bick *et al.* (1998) reported that the mature APS-reductase consists of two distinct domains viz., N- and C-domains and in fact, N-terminal domain of APS-reductase resembles PAPS-reductase while, the C-terminal domain exhibits homology to thioredoxin and acts as a glutaredoxin using reduced GSH as the electron donor. APS-reductase catalyzes a thiol-dependent two-electron reduction of APS to sulfite. There is a great deal of evidence indicating that APS-sulfotransferase is a prime regulation point in SO_4^{2-} assimilation (Brunold and Rennenberg 1997). In fact, vast literature is available regarding situation specific changes in the activity of this enzyme in a variety of plant species after S-starvation (Gutierrez-Marcos *et al.* 1996; Takahashi *et al.* 1997), exposure to reduced S-compounds, heavy-metal stress (Heiss *et al.* 1999; Lee and Leustek 1999), or other stresses. Heavy metals induce the synthesis of phytochelatins, and high concentrations of metal ions significantly increase the demand for cysteine. Recent studies indicate that one potential mechanism for regulating APS-sulfotransferase activity may involve changes in the steady-state mRNA level (Leustek and Saito 1999). Importance of APS-sulfotransferase in sulfate assimilation is due to the fact that this enzyme involves changes in the steady-state mRNA level; while on the other hand sulfite reductase does not appear to be appreciably regulated at the mRNA level (Bork *et al.* 1998). However, further studies are required to know the extent of the abundance of mRNA that

regulates the changes in APS-sulfotransferase activity in plants under normal and under stress.

Detailed information regarding APS-reductase came through genetic studies. The amino acid sequence of plant APS-reductase revealed a multidomain composition (Leustek *et al.* 2000; Suter *et al.* 2000). It is synthesized as a precursor with an amino terminal plastid transit peptide. The amino terminal domain of the mature protein is homologous to PAPS-reductase and the C-terminal domain is homologous to thioredoxin, a redox enzyme. APS-reductase is able to use GSH or dithiothreitol (DTT) as a source of electron. APS-reductase is thought to be one of the key regulators of the sulfate reduction pathway. Its activity and steady-state mRNA level increased markedly and co-ordinately in response to sulfate starvation (Gutierrez-Marcos *et al.* 1996; Takahashi *et al.* 1997; Yamaguchi *et al.* 1999), oxidative stress and/or exposure to heavy metals (Heiss *et al.* 1999; Leustek *et al.* 2000). The oxidative stress and heavy metal exposure have been shown to increase the demand for GSH, and hence, the cysteine necessary for GSH synthesis (Hesse *et al.* 2004). However, the other sulfate assimilatory enzymes are also regulated but to a lesser degree (ATP-sulfurylase) or are constitutively expressed SiR (Bork *et al.* 1998). Vauclare *et al.* (2002) reported that excess feeding of plants with cysteine and GSH decreases the activity of APS-reductase and the level of transcripts; implying that increased internal cysteine and GSH levels might control sulfate assimilation. Further, it can be concluded from the split-root experiments that GSH, and not cysteine, is acting as a signal (Lappartient and Touraine 1996; Hesse *et al.* 2004).

2.7.4.1.3 Sulfite Reductase

Sulfite reductase catalyzes the transfer of six electrons from ferredoxin to sulfite to produce sulfide. The sulfite reductase found in plant cells consists of a homo-oligomer containing a siroheme and an iron-sulfur (Fe-S) cluster per subunit. Sulfite reductase is localized in plastids of both photosynthetic and non-photosynthetic tissues. Electrons are supplied to ferredoxin from PSI in photosynthetic cells and from NADPH in nonphotosynthetic cells. The proper combination of different isoforms of ferredoxin, ferredoxin-$NADP^+$ reductase, and sulfite reductase is critical for efficient sulfite reduction (Yonekura-Sakakibara *et al.* 2000). In fact, sulfite reductase completes the

reduction of S with using electrons donated from reduced Fd. The formed sulfide is incorporated in to cysteine, catalyzed by OAS-TL, with O-acetylserine as substrate.

2.7.4.1.4 O-acetylserine (thiol) lyase or Cysteine synthase

O-acetylserine (thiol) lyase [OAS-TL, EC 4.2.99.8], a key enzyme of plant S metabolism, catalyzes the formation of cysteine from sulfide and O-acetylserine. In fact, the cysteine biosynthetic pathway involves several enzymatic reactions (Brunold and Rennenberg 1997; Leustek and Saito 1999). The SO_4^{2-} is reduced to SO_3^{2-} and then sulfide (S^{2-}) through the sulfate reduction pathway. The final step of cysteine biosynthesis is the incorporation of S^{2-} into cysteine. The reaction is catalyzed by cysteine synthase, which uses S^{2-} and O-acetylserine as the substrates. This final step of cysteine biosynthesis seems to exist necessarily in three major compartments of plant cells, e.g. cytosol, chloroplasts, and mitochondria, since the presence of cysteine synthase has been demonstrated in these three compartments from several plants (Brunold and Suter 1989; Lunn *et al.* 1990). In fact, the metabolic pathways involved in the biosynthesis of cysteine are regulated with a high degree of complexity. Availability of o-acetyl serine, synthesized by serine acetyl transferase (SAT), is generally regarded as a limiting factor and a positive signal for S assimilation and cysteine biosynthesis (Rennenberg 1983). A further level of control is also provided by the formation of a bi-enzyme complex between OAS-TL and SAT, in which the properties of the two enzymes are drastically modified, and the stability of which is dependent on the availability of the OAS-TL substrates, OAS and sulfide (Droux *et al.* 1998; Leustek and Saito 1999). Under non-stressed conditions, overproduction of OAS-TL in plants seems to have less significant effects on the level of the non-cellular thiols than overproduction of SAT. It is not surprising considering the fact, that in *Pisum sativum* in vivo, OAS-TL is present in a huge molar excess over SAT in all compartments (Ruffet *et al.* 1995; Droux 2003). In fact, it is unclear how to explain the observed increase of thiol levels in *Nicotiana tabaccum* OAS-TL transformants without modification of the current models. One possibility is that the OAS-TL/SAT ratio in *N. tabaccum* might be much lower than in *P. sativum*. Nevertheless, under stress conditions, OAS-TL overproducing transformants contain more thiols and are more

tolerant to stress than the control plants suggesting that the increased potential for cysteine synthesis in such conditions still give them an important advantage.

2.7.4.1.5 Serine Acetyltransferase

This enzyme catalyzes the formation of OAS from serine and acetyl CoA. In fact, SAT is responsible for the entry step from serine metabolism to cysteine biosynthesis. A large number of reports are available in literature regarding isolation of cDNA clones encoding SAT from plant species viz., *Citrullus vulgaris* (Saito *et al.* 1995), *Spinacea oleracea* (Noji *et al.* 2001), *A. thaliana* (Bogdanova *et al.* 1995; Hell and Bogdanova 1995; Ruffet *et al.* 1995; Roberts and Wray 1996; Howarth *et al.* 1997) and *Allium tuberosum* (Urano *et al.* 2000). Depending upon the sub-cellular localization, three isoforms of SAT enzyme have been reported. These SAT isoforms were designated as SAT-c (cytoplasmic isoform), SAT-p (plastidic isoform) and SAT-m (mitochondrial isoform). cDNAs of these three SAT isoforms have been cloned from *A. thaliana* (Noji *et al.* 1998).

SAT is one of the major regulatory factors in the biosynthesis of cysteine in plants. In fact, the feedback inhibition of SAT activity by various cysteine concentrations has been reported to regulate the biosynthesis of cysteine in plants (Hell and Bogdanova 1995; Saito *et al.* 1995; Roberts and Wray 1996; Noji *et al.* 1998). However, the inhibition of SAT activity depends upon the sub-cellular isoforms of SAT and plant-specific SAT (Saito *et al.* 1995; Urano *et al.* 2000; Noji *et al.* 2001).

Noji *et al.* (1998) reported in plants that there are two types of SAT that differ in their sensitivity to the cysteine inhibition. Difference of sensitivity to cysteine means that SAT has a regulatory role through the feedback inhibition in cysteine biosynthesis and that depends on the sub-cellular compartmentation (Noji *et al.* 1998; Saito 2000).

2.7.4.1.6 SAT- OAS-TL Bi-enzyme Complex and Cysteine

Two pathways complete the cysteine biosynthesis in plants: the pathway of transport, activation and reduction sulfate into sulphide, and the pathway supplying amino acid moiety, which is derived from serine through OAS, and then yielding cysteine by the reaction of incorporating sulphide moiety into β-position of alanine (Leustek and Saito 1999; Saito 1999; 2000; Hawkesford and Wray 2000; Leustek *et al.*

2000). SAT and OAS-TL are the enzymes committing to the final step of this pathway (Noji and Saito 2003).

Distinct isoforms are localized in plastids, the cytosol and in mitochondria (Hesse *et al.* 1999; Saito 1999). However, the multimeric property of SAT is known, further, there are reports that SAT may form a complex in association with OAS-TL i.e. SAT-OAS-TL bi-enzyme complex (Nakamura *et al.* 1988; Nakamura and Tamura 1990; Bogdanova and Hell 1997; Droux *et al.* 1998; Noji and Saito 2003). Within the bi-enzyme complex, SAT is enzymatically active, whereas OAS-TL is not, but the excess amount of OAS-TL has been shown to catalyze the incorporation of sulfide to form cysteine. The findings suggest that free OAS-TL is responsible for cysteine synthesis and that it also functions as a regulatory subunit of SAT (Leustek *et al.* 2000). Droux *et al.* (1998) reported that the ratio of OAS-TL to SAT in chloroplasts is 300:1, so the majority of OAS-TL is in free form. Berkowitz *et al.* (2002) reported that OAS gets accumulated and allosterically disrupts the bi-enzyme complex under sulfide-limitation and as a result SAT gets inactivated. However, when the S assimilation activity is induced, the OAS level decreases and the bi-enzyme complex is resumed back (Rausch and Wachter 2005).

One of the factors that regulate GSH biosynthesis is cysteine availability, because exogenous addition of cysteine has been shown to increase the GSH content (Farago and Brunold 1994). In addition, thiol group of cysteine is of great importance. It is highly reactive and the cysteine residue present in active centre plays critical roles in the catalytic function of some proteins, so-called SH proteins.

2.8 Sulfur Nutrition and General Plant Growth and Development

Among the plant nutrients, S is of great importance and is required by plants for maintaining normal growth and development and is an essential element required in higher quantity by mustard (Zhao *et al.* 1993; Lakkinani and Abrol 1994; McGrath and Zhao 1996; Scherer 2008). The dry matter of S in plants is only about one-fifteenth of that of N (Saito 2004). A sufficient S supply improves photosynthesis and growth (Ahmad *et al.* 2005; Khan *et al.* 2005; Anjum *et al.* 2008) through regulating N assimilation (Reuveny *et al.* 1980; Scherer 2001; Kopriva *et al.* 2002; Nazar *et al.* 2008).

Sulfur deficiency in the soil is increasing globally due either to the use of high-analysis low-S containing fertilizers, the decreasing use of S-containing fungicides and pesticides, high yielding varieties, intensive agriculture and/or to the reduction of SO_2 emission from industrial sources (Scherer 2001; Eriksen *et al.* 2004). Sulfur deficiency has been shown to influence the plant growth, development (Ahmad *et al.* 2005; Anjum *et al.* 2008; Nazar *et al.* 2008), productivity and quality of many crops, including glucosinolate levels in oilseed rape (Zhao *et al.* 1993), baking quality of *Triticum aestivum* flour (Zhao *et al.* 1999), nutritional value of legume storage proteins (Gayler and Sykes 1985; Spencer *et al.* 1990) and herbage quality of grassland (Murphy and Donnell 1989). In almost 30 field experiments, Zhao *et al.* (1997) found that the glucosinolate concentration of rapeseed was normally higher when grown at S sufficient as compared to S-deficient sites. A large number of studies have reported a marked influence of applied S on the yields of several cereals, pulses, oilseeds, vegetables, forages and other crops as reported in numerous reviews and recent publications (Biswas and Tewatia 1991; Pasricha and Aulakh 1997; Ahmad *et al.* 1998; Jaggi and Dixit 1999; Sarkar 2000; Singh 2001; Aulakh 2003). *Brassica* species are particularly sensitive to S deficiency because they have a high demand for S (Scherer 2001; Ahmad *et al.* 2005; Nazar *et al.* 2008). For example, *Brassica* species produces seeds with a high yield of protein with relatively large quantities of S-containing amino acids (Zhao *et al.* 1997), and the plants require S for the synthesis of glucosinolates, a group of thioglucoside compounds reported to be part of the plant defense mechanism against fungi and insects (Chew 1988). Sulfur availability has been shown to influence lipid, RNA and fatty acid content in developing seeds of *Brassica campestris* (Ahmad and Abdin 2000). They applied S either as single dose or the same dose in split form (in to two or three) portion. S application in three portions increased the oleic acid (18:1) content, and thus improved the quality of soil. The ratio of erucic acid to oleic acid (22:1/18:1) was found closely related to the N:S in the seeds. Thomas *et al.* (2000) studied the effect of effect of S-deficiency on the growth and metabolism of *Beta vulgaris* cv. Druid. Both total S and sulfate concentrations were found markedly reduced in response to S-deficiency, while significant increases in arginine concentration in shoot tissue were observed. Increases were also observed in shoot N

and nitrate concentrations and both shoot and root N/S ratios. Fitzerald *et al.* (2001) studied the effect of S availability (0, 50 and 200 μM) on delivery and metabolism of S in developing endosperm of *T. aestivum*. Application of 200 μM S enhanced the in vitro rates of ATP sulfurylase and OAS-TL. In a number of field studies, *Brassica napus* has found with poor growth and to produce lower seed yield with lower range S levels (Grant *et al.* 2001). Gokhale *et al.* (2005) conducted a field experiment and studied the influence of sources and levels of S on seed yield, quality and S uptake in *Glycine max*. They observed that seed yield, oil content and S uptake significantly increased with increasing levels of S from 0-40 kg/ha. Duhoon *et al.* (2005) reported in *Sesamum indicum* that application of 15 kg S/ha through gypsum or single superphosphate resulted in higher seed and oil yields with higher benefit cost ratio. Ahmad *et al.* (2001) performed the biochemical evaluation of S and N assimilation potential of *B. juncea* under application of sulfur glass fritz, a slow release S-fertilizer. Growth as indicated by biomass accumulation slowed down in response to the application of sulfur glass fritz. A similar trend was observed in the case of photosynthesis rate. The activity of two marker enzymes, ATP-sulfurylase and nitrate reductase, showed very low levels of activity, indicating poor assimilation of S and N by the plant under sulfur glass fritz. They further, concluded that the release of S by sulfur glass fritz is too slow and that the initial non-availability of S to the plants could lead to sub-optimization of both S- and N-assimilating enzymes and that these factors may contribute to low rates of photosynthesis and poor plant growth. Ahmad *et al.* (2005) reported that application of S fertilizer in split doses during growth stages is better than application of the entire amount of S at any stage for obtaining optimum yield of rapeseed. Lakkineni and Abrol (2008) assessed and compared the S requirement of two oilseed species, *B. campestris* and *Arachis hypogea*, and *Triticum aestivum*, a cereal. They studied the S-accumulation pattern in different plant parts at various growth stages and found that compared to two other species, rapeseed-mustard indicated a several-fold increase in S requirement. A low N: S ratio found in rapeseed-mustard also indicated its higher S requirement. They further argued that the additional S required by rapeseed-mustard may be attributed to the presence of glucosinolates, a characteristic of cruciferous plants.

2.9 Nutrition for Sustainable Agriculture under Abiotic Stress Conditions

The sustainability of a cropping system is primarily a function of crop yield and the associated fertility status of the soil (Sogbedi *et al.* 2006). Soil salinity is in fact, one of the major degradation factors for cultivated soils in the world (Dudal 1982; Gupta and Abrol 1990; Lal 1990; Clark and Baligar 2000, Khan *et al.* 2009b) which has been shown to lower the fertility and productivity of many cultivated soils. The poor productivity of crops grown in acid and salt affected soils is mainly due to combinations of elemental toxicities and deficiencies and/or unavailability of essential nutrients (Fageria *et al.* 2008).

The use of nutrient management strategies in improving plant growth and development, achieving optimum yield and in the amelioration of abiotic stress-effects in economically important crop plants growing in varied environmental conditions have been extensively investigated and reviewed (Heiss *et al.* 1999; Lee and Leustek 1999; Cakmak 2005; Hassan *et al.* 2005a, b; Anjana *et al.* 2006; Khan *et al.* 2006, 2007a; Polesskaya *et al.* 2006; Anjum *et al.* 2008; Khan *et al.* 2008). Mineral nutrition alone has contributed significantly to increased crop yields during the 20th century (Fageria *et al.* 2008). In many field studies, horticulturists and agronomists set out to test the hypothesis that N-fertilizer additions alleviate, at least to some extent, the deleterious effect of salinity on plants. Despite the lack of evidence indicating that N applied to saline soil or media above a level considered optimal under non-saline conditions improves plant growth or yield, a number of laboratory and greenhouse studies have shown that salinity can reduce N-accumulation in plants (Pessarakli 1991). Polesskaya *et al.* (2006) investigated the response of *T. aestivum* salinity stress with and without N. Depending on the conditions of N nutrition, salt stress was accompanied by diverse changes in the activity of antioxidant enzymes in the leaves and roots. Resistance of leaves of plants to NaCl-induced oxidative stress correlated with a considerable increase in the activities of APX and GR. Thus, *T. aestivum* plants grown on the NH_4^+-containing medium were found more resistant to the development of oxidative stress in the leaves than those supplied with nitrate as N source. Salt stress did not induce any increase in MDA content in NH_4^+ supplied plants. Irshad *et al.* (2008) studied the

comparative effect of N sources on *Zea mays* under saline and non-saline conditions. All N sources [(urea-N, nitrate-N, 1/2 urea-N + 1/2 nitrate-N (mixed-N), each were applied, each at the rate of 100 kg/ha] greatly stimulated crop growth and nutrient uptake compared with the control. The biomass (shoot and root) of *Z. mays* was significantly greater in mixed-N treatment than in single sources in saline soil whereas it varied in the order of urea-N > mixed-N > nitrate-N > control in non-saline soil. Under both soil conditions, the concentration of Ca, Mg and Na in shoot was highest in nitrate-N treatments while that of K was highest in the control. Shoot N concentration was not significantly different among N sources under non-saline treatment, whereas under saline conditions, the concentration varied markedly in the order of nitrate-N > urea-N > mixed-N > control. The mineral concentrations in the shoot increased under salt treated soil when compared with non-saline soil. The ratios of Na/K, Na/Ca and Na/Mg were also higher under salt stress due to higher accumulation of Na ion in the shoot. Among N-fertilizer sources, Na/Ca and Na/Mg ratios were highest in control whereas Na/K ratio was the highest in nitrate-N treatment. The lowest cation ratios were noted in mixed-N-treated plants under both soils.

2.10 Sulfur Nutrition and Plant Abiotic Stress Tolerance

Sulfur plays essential roles in various important mechanisms such as Fe/S clusters in enzymes, vitamin cofactors, GSH in redox homeostasis and detoxification of xenobiotics (Leustek *et al.* 2000; Saito 2000). Reduced S incorporated in cysteine and methionine amino acids plays essential roles in catalytic centers and disulfide bridges of proteins (Hell 1997). Cysteine can be directly utilized for protein synthesis or further metabolized to Met, coenzymes or GSH. The tripeptide GSH is the most abundant low molecular weight thiol in plants and thus, the main storage and transport form of reduced S and, GSH plays an important role in the defense against various biotic and abiotic stress conditions and in redox buffering of the cell (May *et al.* 1998a, b; Noctor *et al.* 1998a, b; Anjum *et al.* 2008; Srivalli and Khanna-Chopra 2008). The GSH synthesis in plants has been found to be dependent on and regulated by the S supply of the plants. During S deficiency GSH content rapidly decreased in tobacco cell cultures but was rebuilt upon the re-supply of sulphate and/or cysteine (Smith 1980). Glutathione is reduced in plants subjected to S deficiency (Nikiforova *et al.* 2003). Ruiz

and Blumwald (2002) studied the role of S assimilation and the biosynthesis of cysteine and GSH during the response to salt stress of wild type and salt-tolerant transgenic *Brassica napus* L. (canola) plants over expressing a vacuolar Na^+/H^+ antiporter. They observed a 3-fold increase in cysteine and GSH content in wild type plants exposed to salt stress, but not in the transgenic plants. The induction of cysteine and GSH synthesis during salt stress in the wild type plants suggested a possible protective mechanism against salt-induced oxidative damage. On the other hand, the salt-tolerant transgenic plants did not show significant changes in either cysteine or GSH content, and thus confirmed the role of vacuolar Na^+ accumulation and ion homeostasis in salt tolerance. Hassan *et al.* (2005a, b) studied the role of S levels in the alleviation of Cd-caused growth inhibition and oxidative stress in *O. sativa*. In comparison with the lower S level (0.2 mmol), the higher S levels (0.4 and 0.6 mmol) helped alleviate Cd toxicity, characterized by a significant increase in growth parameters, and a decrease in Cd^{2+} and MDA content in both roots and shoots. Khan *et al.* (2007a) studied in Cd-treated *T. aestivum* cultivars (PBW343 and WH542) the activities of antioxidative enzymes and ATP-sulfurylase, the rate limiting enzyme in S assimilation in plants, to assess their involvement in determining yield potential, and to understand the integral physiological response of wheat cultivars. They found that the high yield potential of PBW343 was the result of the efficiency of the antioxidative enzyme system and greater capacity of S assimilation than WH542. Under Cd stress PBW343 showed increased activities of antioxidative enzymes, greater capacity of S assimilation and removed ROS efficiently. These characteristics of PBW343 helped in protecting the photosynthetic apparatus maintaining high growth and yield. The low yielding potential cultivar WH542 experienced greater oxidative stress under Cd stress and showed higher SOD activity to convert superoxides to H_2O_2 but had poor capacity of other antioxidative enzyme system and S assimilation, and resulted in lower photosynthesis, growth and yield. Supplementary S fertilization to high S loving crops such as *Brassicas* and leguminous crops have been to enhance plant-stress-defence operations and act indirectly by improving general plant performance under abiotic and biotic stresses as well through improving GSH and AsA (Rausch and Wachter 2005). Based on the very interesting relationships between AsA and GSH pools with net photosynthesis and plant dry mass

with and without S, Anjum *et al.* (2008) suggested that adequate S supply may improve the pools of these compounds in plants to a great extent that may lead to increase in photosynthetic efficiency and subsequently to plant dry mass and crop yield. Ruiz and Blumwald (2002) studied the role of S assimilation and the biosynthesis of cysteine and GSH in wild type and salt-tolerant transgenic (over expressing a vacuolar Na^+/H^+ antiporter) *B. napus* plants treated with salinity levels. A 3-fold increase in cysteine and GSH content was observed in wild type plants exposed to salt stress, but not in the transgenic plants. They further argued that the induction of cysteine and GSH synthesis during salt stress in the wild type plants may suggest a possible protective mechanism against salt-induced oxidative damage. On the other hand, the salt-tolerant transgenic plants did not show significant changes in either cysteine or GSH content, confirming the role of vacuolar Na^+ accumulation and ion homeostasis in salt tolerance. They concluded that in wild-type *Brassica* plants, salt stress induced an increase in the assimilation of S and the biosynthesis of cysteine, and GSH aimed to mitigate the salt-induced oxidative stress.

2.11 Critical Appraisal of the Review and Rationale of the Present Study

The picture which emerges from reviewing the available literature is that a considerable information has been accumulated regarding the plant responses to salinity stress but the role of mineral nutrients status of plants in the regulation of plant responses to salinity stress in economically important crops including mustard is still in its infancy where the detailed studies need to be carried out for commenting on the role of S nutrition in salinity stress tolerance which has not been well documented.

Elucidation of the responses of plants differing in nutrient accumulation /transport index in terms of changes in plant growth, photosynthetic functions, components of antioxidant defense system, utilization pattern of nutrient(s) applied and the yield and its closely related attributes is thus, expected to accelerate progress in our understanding of strategies adopted by these plants while grown under salinity stress. In addition, the studies on the role of S availability, timing of S application on S-mediated protection against salinity-induced alteration in growth, photosynthesis, oxidative stress and productivity in mustard genotypes thus, may further strengthen our understanding.

Keeping above facts in mind the work reported in the present thesis was undertaken with the assumption that mustard type with high ATP-sulfurylase activity and thus high S accumulation capacity will be more tolerant to salinity stress. Further, the cultivars differing in ATP-sulfurylase activity (S accumulation capacity) will respond differentially to S application under salinity stress.

Table 1: Distinguishing features of saline and sodic soils.
(Modified after Abrol *et al.* 1988)

Characteristics	Saline soils	Sodic soils
Chemical	• Dominated by neutral soluble salts consisting of chlorides and sulphates of sodium, calcium and magnesium.	• Appreciable quantities of neutral soluble salts generally absent. Measurable to appreciable quantities of salts capable of alkaline hydrolysis, e.g. Na2CO3, present.
	• pH of saturated soil paste is less than 8.2.	• pH of the saturated soil paste is more than 8.2.
	• An electrical conductivity of the saturated soil extract of more than 4 dS/m at 25 °C is the generally accepted limit above which soils are classed as 'saline'.	• An exchangeable sodium percentage (ESP) of 15 or more is the generally accepted limit above which soils are classed as 'sodic'. Electrical conductivity of the saturated soil extract is generally less than 4 dS/m at 25 °C but may be more if appreciable quantities of Na2CO3 etc. are present.
	• There is generally no well-defined relationship between pH of the saturated soil paste and exchangeable sodium percentage (ESP) of the soil or the sodium adsorption ratio (SAR) of the saturation extract.	• There is a well defined relationship between pH of the saturated soil paste and the exchangeable sodium percentage (ESP) of the soil or the SAR of the saturation extract for an otherwise similar group of soils such that the pH can serve as an approximate index of soil sodicity (alkali) status.
	• Although Na is generally the dominant soluble cation, the soil solution also contains appreciable quantities of divalent cations, e.g. Ca and Mg.	• Sodium is the dominant soluble cation. High pH of the soils results in precipitation of soluble Ca and Mg such that their concentration in the soil solution is very low.
	• Soils may contain significant quantities of sparingly soluble calcium compounds, e.g. gypsum.	• Gypsum is nearly always absent in such soils.
Effect on plant growth	• In saline soils plant growth is adversely affected: - chiefly through the effect of excess salts on the osmotic pressure of soil solution resulting in reduced availability of water; - through toxicity of specific ions, e.g. Na, Cl, B, etc.;	• In sodic soils plant growth is adversely affected: - chiefly through the dispersive effect of excess exchangeable sodium resulting in poor physical properties; - through the effect of high soil pH on nutritional imbalances including a deficiency of calcium; - through toxicity of specific ions, e.g. Na, CO3, Mo, etc.

Material and Methods

Contents

MATERIAL AND METHODS

This chapter deals with the description of material used for the study and methods adopted for the experimentation and determination of various traits during the course of the investigation.

3.1 Plant Material

The experimental material selected for the study was mustard (*Brassica juncea* L. Czern & Coss.). Mustard cultivars, 'Alankar', 'Varuna', 'Pusa Jai Kisan' and 'SS2' were obtained from the National Research Centre on Plant Biotechnology (NRCPB) of the Indian Agricultural Research Institute (IARI), New Delhi, India.

3.1.1 Mustard

3.1.1.1 Nomenclature

The oleiferous *Brassica* grown in India is divided into four groups:

1. Brown mustard: Commonly called rai, raya or laha (*Brassica juncea* L. Czern & Coss.)
2. Sarson
    a. Yellow sarson: *Brassica campestris* L. var. Sarson Prain
    b. Brown sarson: *Brassica campestris* L. var. Dichotoma Watt
3. Toria: lahi or maghi lahi *Brassica campestris* L. var. Toria Duth
4. Taramira or Tara (*Eruca sativa* Mill.)

In trade, sarson, toria and taramira are known as rapeseed, and rai as mustard.

In addition, there are two other species, namely *Brassica nigra* Koch. (Banarasi rai) and *Brassica juncea* var. Rugosa (Pahadi rai). These two species do not fall under any of the four groups. These are, moreover, grown to a limited extent. Mustard (*Brassica juncea* L. Czern & Coss.) is the dominant species grown in India (Prakash 1980; Yadava and Singh 1999).

3.1.1.2 Botanical Description

Rape and mustard include annual herbs. Roots, in general, are long and tapering. Toria is more or less a surface feeder but Brown sarson bears long roots with limited lateral spread enabling its successful cultivation under drier conditions. The height of the stem varies from 45 cm (in some varieties of Toria) to 190 cm (in Yellow

sarson). In Toria and Brown sarson, the branches arise at an angle of 30° to 40°. In Yellow sarson, the branches arise laterally at an angle of about 10° to 20° and give the plant a narrow and pyramidal shape. The inflorescence is a corymbose raceme. In the case of Yellow sarson, the four petals are spread apart, whereas, in Brown sarson and Toria, the petals overlap or may be placed apart, depending upon the cultivar. The flowers bear a hypogynous ovary. In Brown sarson and Toria, the ovary is bicarpellary, whereas in Yellow sarson, it may also be tri-or tetra-carpellary. The fruit is sliliqua. The sliliqua are two-valved, three-valved or four-valved, depending upon the number of carpels in the ovary. The flower begins to open from 8:00 h and continue up to 12:00 noon.

3.1.1.3 Genomic Relationships of *Brassica juncea*

The relationship between the *Brassica* species was established by cytological studies in the 1930's (Morinaga 1934). Each of the three diploid species represented a parental genome of the amphidiploid species. *B. rapa* contains the A-genome (n=10), *B. nigra* contains the B-genome (n=8) and *B. oleracea* contains the C-genome (n=9) and these are combined in *B. juncea* (A+B genome, n=18), *B. napus* (A+C genomes, n=19) and *B. carinata* (B+C genomes, n=17) (Figure 2).

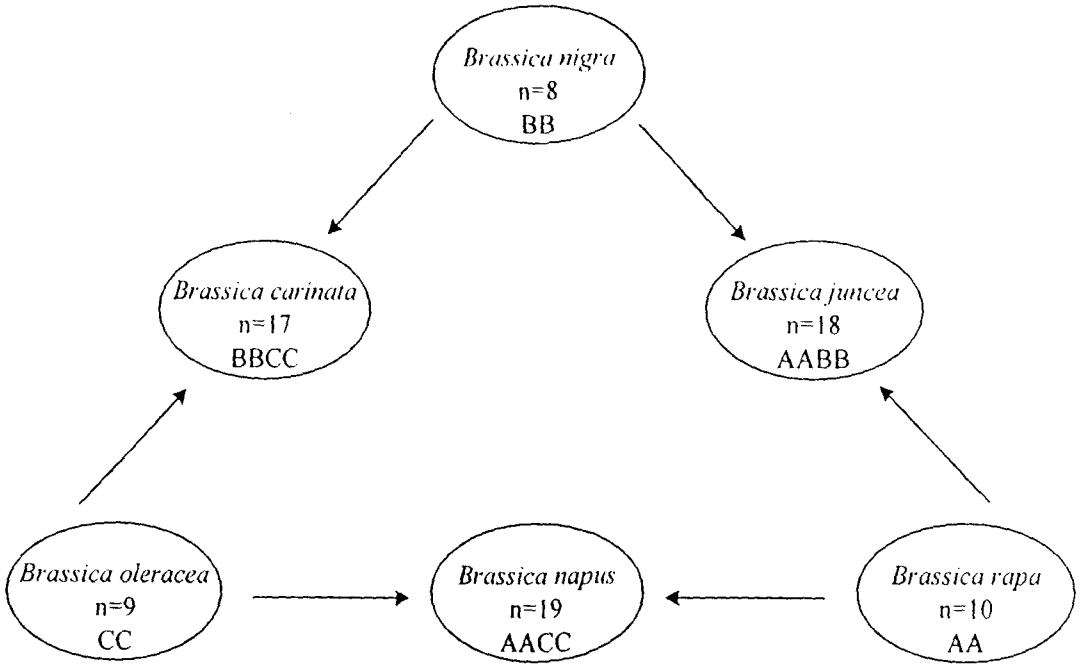


Figure 2. Genomic relationships between *Brassica* species (Arranged according to U 1935).

The relationships have later been confirmed by morphological, biochemical and restriction fragment length polymorphism (RFLP) studies (Mithen *et al.* 1984; Takahata and Hinata 1986; Song *et al.* 1988). Amphidiploid species have also been resynthesized by interspecific crossing of the parental genotypes (Olson and Ellestorm 1960) and by protoplast fusions (Glimelius 1999).

3.2 Climatic Conditions of Aligarh

Aligarh has an area of about 5,024 sq kms, situated at 27°52'N latitude, 78°51'E longitude, and 187.45 m altitude above sea level. It has severe hot dry summers and intense cold winters prevail during the year. The winter extends from the middle of October to the end of March. The mean temperature for January, the coldest month, is about 13°C. The extreme minimum recorded for any single day is 0.5°C. The summer extends from April to the end of June and the average temperature for June is about 34°C, whereas the extreme maximum record is 45.5°C. The monsoon extends from the end of June to middle of October. The mean annual rainfall is about 847.3 mm. More than 85% of the total rainfall occurs during June to September and some 10% useful for 'Rabi' (winter) crops. The relative humidity in the winter ranges between 56% and 77% with an average of 66.5% and that of summer, 37% to 49% with an average of 43%; and that of the monsoon season, between 63% and 73% with an average of 68%.

3.3 Experimentation

The experiments were conducted in plastic pots filled with acid-washed sand during the winter season of 2005, 2006 and 2007 on mustard (*Brassica juncea* L. Czern & Coss.) in the greenhouse of the Department of Botany, Aligarh Muslim University, Aligarh, India under natural day/night conditions.

3.3.1 Sand Culture

3.3.1.1 Purification of Sand

Before the beginning of each experiment, sand was purified (Hewitt, 1966). First of all the coarse sand was washed thoroughly with tap water, then treated with 18% HCl for 24 h followed by washing with de-ionized water and drying the sand completely. The acid-washed sand was used for filling 23-cm diameter pots. Two plants per pot were maintained and fed with 250 ml of Hoagland nutrient solution

(Hewitt, 1966) every alternate day and 200 ml of de-ionized water daily. The nutrient solution was replaced weekly.

3.3.2 Preparation of Nutrient Solution

Hoagland Nutrient solution for plant culture

Solution 1 contained following salts (g/l)

KH_2PO_4	:	0.136
KNO_3	:	1.02
$Ca(NO_3)_2$	:	0.492
$MgSO_4.7H_2O$	:	0.49

Solution 'A' contained following salts (mg/l)

H_3BO_3	:	2.86
$MnCl_2.4H_2O$	:	1.81
$ZnSO_4.7H_2O$	:	0.22
$CuSO_4.5H_2O$	:	0.08
$H_2MoO_4.H_2O$	:	0.09

Solution 'B'

26.1 g EDTA was dissolved in 268 ml of 1N KOH. To this, 24.9 g $FeSO_4.7H_2O$ was added and diluted to one litre. The solution was aerated overnight to produce stable ferric complex. The pH of the solution was 6.5.

One ml of Solution 'A' was mixed into one litre of Solution 1 and to it 1ml of Solution 'B' was added. The pH of the solution was adjusted to 6 with 0.1N H_2SO_4.

3.3.3 Sowing of Seeds

Healthy seeds of mustard cultivars were surface sterilized with 0.5% (v/v) sodium hypochlorite for 15 min and then rinsed three times with de-ionized water. Before sowing, light application of de-ionized water was given in each pot to provide necessary moisture for germination of seeds. Ten seeds per pot were sown to avoid germination failure and after the establishment of seedlings, thinning was done to retain only two healthy plants of nearly equal size in each pot.

3.4 Experimental Lay-out and Experimentation

The treatments in each experiment were arranged in a randomized block design with three replications. Seeds of mustard were sown in plastic pots containing purified

sand. After the seedling establishment, two plants per pot were maintained. Prior to sowing, 250 ml Hoagland nutrient solution was given to all pots. In order to check the aphid contagion, if any, insecticidal spray of Dimecron-100 was done with a hand spray. Calendar of various operations in each Experiment is given in Table 2, which shows time schedule for experimentation, treatment and the data collection.

Table 2. Experimental calendar showing treatments and sampling for the three experiments.

	Experiment 1 (2005-2006)	Experiment 2 (2006-2007)	Experiment 3 (2007-2008)
Preparation of sand for experiment	01.10.2005	05.10.2006	08.10.2007
Sowing	08.10.2005	10.10.2006	10.10.2007
Treatments	Hoagland nutrient solution	Hoagland nutrient solution + NaCl	Hoagland nutrient solution + NaCl + SO_4^{2-}
Replication	Three	Three	Three
Cultivars	Alankar, Varuna, Pusa Jia Kisan, SS2	Pusa Jai Kisan (Sulfur-efficient) SS2 (Sulfur-inefficient)	Pusa Jai Kisan (Sulfur-efficient) SS2 (Sulfur-inefficient)
Samplings			
Pre-flowering (30 DAS)	07.11.2005	09.11.2006	09.11.2007
Flowering (60 DAS)	07.12.2005	09.12.2006	09.12.2007
Harvest (120 DAS)		09.02.2006	09.02.2007

3.4.1 Experiment 1

Experiment 1 was conducted in the winter season of 2005-2006. Surface sterilized seeds of four mustard cultivars namely, 'Alankar', 'Varuna', 'Pusa Jai Kisan' and 'SS2' were sown on 8th October, 2005 in 23-cm pots containing acid washed sand to which 250 ml Hoagland solution was given once a day in the morning. The treatments in this Experiment were arranged in a complete randomized block design and replicated three times. The aim of the experiment was to select sulfur-efficient and sulfur-inefficient mustard cultivars on the basis of ATP-sulfurylase activity and sulfur

content. Sampling was done at 30 and 60 DAS. There were total 12 pots. Each pot had two plants. One plant from each cultivar and replicate was used for sampling at 30 DAS and another plant was selected for sampling at 60 DAS.

3.4.2 Experiment 2

Experiment 2 was conducted in the winter season of 2006-2007. This experiment was conducted using the sulfur-efficient and sulfur-inefficient cultivars Pusa Jai Kisan and SS2, respectively based on the findings of Experiment 1. The aim of the experiment was to study the influence of 50 and 100 mM NaCl stress on the physiological changes in Pusa Jai Kisan and SS2, and to understand the basis of the difference in salt tolerance of these two cultivars. Plants were fed with 250 ml of Hoagland nutrient solution containing NaCl treatments once in a day in the morning. A control group of plants were included in the study to which NaCl was not given with the nutrient solution. Flushing was done once a week to remove excess NaCl, if any. The experiment was conducted in a factorial randomized block design and each treatment was replicated three times. Seeds of Pusa Jai Kisan and SS2 were sown in 23-cm diameter pots on 10th October, 2006. The cultivars were tested for growth, photosynthesis, leaf water potential and leaf osmotic potential, accumulation of nutrients, ions, enzymatic and non-enzymatic antioxidants and oxidative stress at 30 and 60 DAS. Yield was determined at maturity. Harvesting was done at 120 DAS.

In Experiment 2, number of total pots used for experimentation was 48. For each treatment there were 8 pots. Plants in the three replicates in each treatment were used for sampling at 30 DAS and another three replicates were used for sampling at 60 DAS. The remaining two replicates were used for determining yield. At 30 and 60 DAS sampling time, one plant from each treatment and replicate was selected for photosynthesis measurements (n = 3) and growth (n =3). These plants were dried and accumulation of nutrients and ions (n = 3) was determined in the dried plant material. Another plant of pot in each treatment and replicate was used for study of S assimilation (n = 3), antioxidants and oxidative stress (n = 3). Yield data were collected from the two plant per pot in each treatment and two replicates (n = 4).

3.4.3 Experiment 3

Experiment 3 was conducted in the winter season of 2007-2008. This experiment was also conducted using the sulfur-efficient and sulfur-inefficient cultivars Pusa Jai Kisan and SS2. The aim of the experiment was to study the potential of application of sulfur in the amelioration of salinity stress in the two cultivars differing in sulfur accumulation capacity. Plants were grown with 100 mM NaCl, 1 or 2 mM SO_4^{2-} or with NaCl and sulfur combination (100 mM NaCl + 1 mM SO_4^{2-}, 100 mM NaCl +2 mM SO_4^{2-}). A control group of plants were grown with nutrient solution. $MgSO_4$ was added for obtaining different concentrations of SO_4^{2-} and Mg^{2+} was maintained by the addition of $MgCl_2$. The treatments were arranged in a factorial randomized block design and replicated three times. Sowing of seeds was done on 10th October, 2007. The size of the pots used and the amount of nutrient solution were same as in earlier experiments. The plant characteristics studied and timing of sampling in this experiment were same as for Experiment 2. Harvesting of the crop was done at 120 DAS.

The number of pots used for Experiment 3 was 96 as there were 6 treatments and 2 cultivars. For each treatment there were 8 pots. The use of plant and replicates for sampling at 30 and 60 DAS, and at harvest was same as described for Experiment 2.

3.5 Plant Sampling

As the aim of the Experiment 1 was to identify sulfur-efficient and sulfur-inefficient mustard types, data on ATP-sulfurylase activity and sulfur accumulation were recorded. In addition data on growth and photosynthesis were also recorded at 30 and 60 DAS. In Experiment 2 and 3, data on growth, photosynthesis, water relations (leaf water potential, leaf osmotic potential), accumulation of nutrients and ions, enzymatic and non-enzymatic antioxidants and oxidative stress were recorded at 30 and 60 DAS, and yield at maturity (120 DAS).

3.6 Chemicals

Chemicals used in the study were obtained from Sigma-Aldrich (St. Louis Mo, USA). Other major and minor salts and buffer components were procured from MERK, SRL and/or HIMEDIA.

3.7 Parameters Studied

Following parameters were studied at different sampling times.

3.7.1 Sulfur Assimilation

- ATP-sulfurylase activity
- S content

3.7.1.1 Assay of ATP-sulfurylase Activity

The method of Lappartient and Touraine (1996) was followed for the assay of ATP-sulfurylase activity. The details of the procedure are given below.

3.7.1.1.1 Enzyme Assay

One gram fresh leaf tissue was rapidly ground at 4°C in a buffer consisting of 10 mM Na_2EDTA, 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol (DTT), and 0.01 g/ml insoluble PVP, using a 1:4 (w/v) tissue to buffer ratio. The homogenate was centrifuged at 20,000 × g (CPR24, Remi, New Delhi, India) for 10 min at 4°C. The supernatant (crude extract) was used for the *in vitro* ATP-sulfurylase assay. The enzyme activity was measured using molybdate-dependent formation of pyrophosphate. The reaction was initiated by adding 0.1 ml of crude extract to 0.5 ml of the reaction mixture, which contained 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 units/ml of sulfate-free inorganic pyrophosphatase in 80 mM Tris-HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture except that Na_2MoO_4 was absent. Incubations were carried out side by side at 37°C for 15 min, after which phosphate was determined on a spectrophotometer (SL164, Elico, Hyderabad, India).

3.7.1.2 S Content

3.7.1.2.1 Digestion of Leaf Powder

Oven-dried leaf powder (100 mg) was taken in digestion tube of 75 ml capacity. In digestion tube, 4.0 ml acid mixture (consists of concentrated nitric acid and perchloric acid in the ratio of 1:1) and 7.5 mg of selenium dioxide as catalyst was added. The digestion was carried out till the digested solution became colourless. Following digestion, the volume was made up to 75.0 ml with de-ionized water. The interference of silica was checked by filtering the contents of the tube.

3.7.1.2.2 Estimation of S

Total sulfur in plant samples was estimated according to the turbidimetric method of Chesnin and Yien (1950). A 5 ml aliquot was pipette out from the digested solution for turbidity development in 25 ml volumetric flask. Turbidity was developed by adding 2.5 ml gum acacia (0.25%) solution, 1.0 g $BaCl_2$ sieved through 40-60 mm mesh and the volume was made up to the mark with de-ionized water. The contents of 25 ml volumetric flask were thoroughly shaken till $BaCl_2$ completely dissolved. Turbidity was allowed to develop for 2 min. The values were recorded at 415 nm within 10 min after the turbidity development. A blank was also run simultaneously after each set of determination.

The amount of sulfate was calculated with the help of a calibration curve drawn afresh using a series of K_2SO_4 solutions.

3.7.2 Photosynthetic Characteristics

Following photosynthesis related parameters were studied.

- Net photosynthetic rate
- Stomatal conductance
- Intercellular CO_2 concentration
- Transpiration rate
- Water-use efficiency
- Chlorophyll fluorescence

Net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate were measured on the uppermost fully expanded second leaf of plants placed in a 1 litre leaf chamber of Li6200 portable photosynthesis system (LiCor, Lincoln, Nebraska, USA) at light saturation intensity between 11 and 12 h. The atmospheric conditions during measurement were photosynthetically active radiation (PAR), 850±22 µmol photons/m^2/s, relative humidity 62±3%, atmospheric temperature, 22±1°C and atmospheric CO_2, 360 µmol/mol. The ratio of atmospheric CO_2 to intercellular CO_2 concentration was constant. Water-use efficiency was calculated as the ratio of photosynthesis to transpiration.

Chlorophyll fluorescence parameters, maximal fluorescence (*Fm*), variable fluorescence (*Fv*) and the ratio of *Fv*/*Fm* of fully expanded second leaf from top were

measured *in vivo* after half an hour dark adaptation of the leaves using chlorophyll fluorometer (OS-30p, USA).

3.7.3 Water relations

- Leaf water potential
- Leaf osmotic potential

Water potential is defined as the free energy of water in a system compared with the free energy of pure water at the same temperature and pressure. The total water potential (Ψ) is the sum of its components including the matric potential (Ψ_π), osmotic potential (Ψ_τ) and pressure potential (Ψ_p). Leaf water potential was measured on second leaf from top (fully expanded youngest leaf) of the plant by using water potential system (Psypro, WESCOR, USA).

The same leaf, as used for water potential, was frozen in liquid nitrogen in sealed polythene bags which was thawed and cell sap was extracted with the help of a disposable syringe. The sap so extracted was directly used for the determination of osmotic potential using a vapour pressure osmometer (5520, WESCOR, USA).

3.7.4 Content of nutrients and ions

- Leaf nitrogen content
- Leaf phosphorous content
- Leaf potassium content
- Leaf calcium content
- Leaf sodium content
- Root sodium content
- Leaf chloride content
- Root chloride content

3.7.2 Digestion of Sample for Estimation of Nutrients Content

Oven-dried sample (leaf or root) powder (100 mg) was carefully transferred to a digestion tube and 2 ml of concentrated sulphuric acid was added to it. The contents of the flask were heated on a temperature controlled assembly for about 2 h. As a result, the contents of the tube turned black. It was cooled for about 15 min at room temperature and then 0.5 ml 30% H_2O_2 was added drop by drop and the solution was heated again till the colour of the solution changed from black to light yellow. After

further cooling for about 30 min, additional 3 to 4 drops of 30% H_2O_2 were added, followed by heating for another 15 min. It was repeated till the light yellow colour turned colourless. The digested material was transferred from the tube to a 100 ml volumetric flask with three washings with de-ionized water. The volume of the volumetric flask was then made up to the mark (100 ml) with de-ionized water.

3.7.2.1 Determination of N, P, K and Ca

Determination of N, P and K content in leaf was done in peroxide digested sample. Nitrogen was determined by the method of Lindner (1944), while phosphorus was estimated by the method of Fiske and Subba Row (1925). Potassium and calcium were determined using flame photometer.

3.7.2.1.1 Estimation of N

Leaf nitrogen content was estimated by the Kjeldahl digestion method as described by Lindner (1944).

A 10 ml aliquot of the digested material was taken in a 50 ml volumetric flask. To this, 2 ml of 2.5 N sodium hydroxide and 1ml of 10% sodium silicate solutions were added to neutralize the excess of acid and to prevent turbidity, respectively. The volume was made up to the mark with de-ionized water. In a 10 ml graduated test tube, 5 ml aliquot of this solution was taken and 0.5 ml Nessler's reagent was added. The final volume was maintained with de-ionized water. The contents of the test tubes were allowed to stand for 5 min for maximum colour development. The optical density of the solution was read on a spectrophotometer at 525 nm.

3.7.2.1.1.1 Preparation of Standard Curve

50 mg ammonium sulphate was dissolved in de-ionized water to get 1 litre solution. From this, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml solutions were taken in ten different test tubes. The solution in each test tube was diluted to 5ml with de-ionized water. In each test tube 0.5 ml of Nessler's reagent was added. After 5 min, the intensity of the colour was read at 525 nm. A blank was run simultaneously with each set of determination.

Standard curve was plotted using different concentrations of ammonium sulphate solution versus optical density and with the help of the standard curve, the amount of nitrogen present in the sample was determined.

3.7.2.1.2 Estimation of P

The method of Fiske and Subba Row (1925) was adopted for the estimation of phosphorus. A 5 ml aliquot of the digested material was taken in a10 ml graduated test tube and 1ml of 2.5% molybdic acid reagent was carefully added followed by the addition of 0.4 ml of 1-amino-2 naphthol-4-sulphonic acid. The addition of this solution turned the colour of the contents blue and the volume was made up to 10 ml. The solution was shaken for 5 min for maximum colour development and transferred to a colorimetric tube. The intensity of the colour was read at 620 nm. A blank was run simultaneously.

3.7.2.1.2.1 Preparation of Standard Curve

351 mg monobasic dihydrogen orthophosphate dissolved in sufficient de-ionized water to which 10 ml of 10 N H_2SO_4 was added and the final volume was made up to 1 litre. From this, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml solutions were taken in ten different test tubes. The solution in each test tube was diluted to 5 ml. In each test tube, 1 ml molybdic acid reagent and 0.4 ml of 1-amino-2 naphthol-4-sulphonic acid were added and the final volume was made up to 10 ml. After 5 min, the intensity of the colour was read at 620 nm. A standard curve was plotted using different dilutions of potassium dihydrogen orthophosphate solution versus optical density and with the help of standard curve, the amount of phosphorus present in the sample was determined.

3.7.2.1.3 Estimation of K

It was estimated with the help of flame photometer (Khera-391, New Delhi, India). A 10 ml aliquot was taken and read by using the filter for potassium. A blank was also run side by side with each set of determination. The readings were compared with calibration curve plotted using known dilutions of standard potassium chloride solution.

3.7.2.1.3.1 Preparation of Standard Curve

1.91 g potassium chloride was dissolved in 100 ml of de-ionized water, and 1 ml of this solution was diluted to 1 litre. This represents solution of 10 ppm potassium concentration. From this 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 ml solutions were transferred to 10 vials separately. The solution in each vial was diluted to 10 ml. The diluted solution

of each vial was run separately. A blank was also run with the each set of determination. Standard curve was prepared using different dilutions of potassium chloride solution versus readings on the flame photometer.

3.7.2.1.4 Estimation of Ca

The calcium in digested samples was estimated with the help of the flame photometer.

3.7.2.1.4.1 Preparation of Standard Curve

2.497 g $CaCO_3$ in 15 ml of concentrated HCl was dissolved and the volume was made to 1 litre with de-ionized water. Different dilutions of 0, 100, 200, 300, 400 and 500 ppm calcium were prepared from 1000 ppm calcium solution. The readings were directly read on the flame photometer. A standard curve, taking known dilutions of standard $CaCO_3$ solutions was plotted. The reading of each sample was compared with this calibration curve and calcium in samples was calculated.

3.7.3 Digestion of Sample for Estimation of Ions Content

50 mg of oven-dried leaf and root material was taken in a 50 ml volumetric flask. To this, 2 ml concentrated nitric acid was added and it was heated on an electric hot plate till the appearance of brown effervescence. At the stop of effervescence, Tri acid mixture (TAM) solution was added till a clear solution was obtained. TAM is a mixture of nitric acid, sulphuric acid and perchloric acid mixed in the ratio of 10:5:4. The material was then allowed to dry on hot plate. After drying, 50 ml of de-ionized water was added, shaken and transferred to another 50 ml volumetric flask with three washings. The final volume was made up to mark.

3.7.3.1 Estimation of Na+

Sodium was estimated using flame photometer.

3.7.3.1.1 Preparation of Standard Curve

Standard curve was prepared by taking known concentrations of sodium. 5.845 g of NaCl was dissolved in de-ionized water and the volume was made to 1 litre, that gave 100 milliequivalents (meq) per litre of sodium. Different dilutions of 5, 20, 30, 40 and 50 meq sodium was prepared from the stock solution. The concentrations of sodium in the unknown sample were read from the graph plotted with the readings of flame photometer and dilutions.

3.7.3.2 Estimation of Cl-

50 ml of digested sample was taken in a flask and 2 ml of 5% K_2CrO_4 indicator was added. It was titrated against 0.02 N silver nitrate solution and chloride content was calculated as follows:

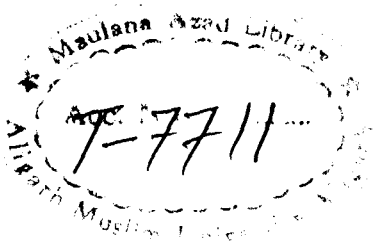
Chloride (mg/l) = (A-B) N of AgNO3 × 1000 × 35.5/ml sample

Where A= ml titration for sample

B= ml titration for blank

3.7.4 Oxidative Stress

- Thiobarbituric acid reactive substances content
- H_2O_2 content
- Electrolyte leakage
- Membrane stability index
- Relative salt injury



3.7.4.1 Determination of Thiobarbituric Acid Reactive Substances Content

The level of lipid peroxidation products in the leaves was determined by thiobarbituric acid reactive substances (TBARS) as described by Dhindsa *et al.* (1981). Fresh leaf tissue (200 mg) was ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using mortar and pestle. After heating at 95°C for 30 min, the mixture was quickly cooled on ice bath and centrifuged at 10,000×g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The blank was also run. The TBARS content was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}).

3.7.4.2 Determination of Hydrogen Peroxide Content

The content of H_2O_2 in leaves was measured as described by Jana and Chaudhuri (1981). Fresh leaves (100 mg) were homogenized with 6.0 ml of K-phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000×g for 25 min. To 3 ml of the extracted solution, 1 ml of 0.1% titanium sulfate in 20% H_2SO_4 was added and then centrifuged at 6,000 × g for 15 min. The colour intensity of the supernatant was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient (0.28 $\mu mol^{-1}cm^{-1}$).

3.7.4.3 Electrolyte Leakage

For measuring electrolyte leakage, samples were thoroughly washed with sterile water to get rid of surface-adhered electrolyte and kept in closed vials containing 10 ml of de-ionized water and incubated at 25°C for 6 h on a shaker and consequently electrical conductivity was determined (C_1). Samples were then again kept at 90°C for 2 h and the electrical conductivity was obtained after attaining equilibrium at 25°C (C_2). Electrolyte leakage was calculated by using the following equation.

$$\text{Electrolyte leakage (\%)} = (C_1/ C_2) \times 100$$

3.7.4.4 Membrane Stability Index

Leaf membrane stability index (MSI) was determined according to the method of Premachandra *et al.* (1990) and modified by Sairam (1994). Small discs from the leaves were cut, weighed (0.2 g) and kept in 10 ml of de-ionized water at 40°C for 30 min. After incubation electrical conductivity of the water containing the sample was measured (C_1). Test tubes in the second set were incubated at 100°C for 15 min and electrical conductivity was measured as above (C_2) and MSI was calculated.

$$\text{Membrane Stability Index (MSI)} = [1- (C_1/ C_2)] \times 100$$

3.7.4.5 Relative Salt Injury

0.5 g leaf sample was washed with de-ionized water then placed in a test tube containing 12 ml of de-ionized water, kept at 27°C, and then its electrical conductivity was recorded (C_1). The same was autoclaved for 10 min and then cooled to room temperature and electrical conductivity (C_2) was recorded and relative salt injury was calculated.

$$\text{Relative Salt Injury (RSI)} = [EC_1/ (EC_1+EC_2)] \times 100$$

3.7.5 Enzymatic and Non-enzymatic Antioxidants

- Superoxide dismutase activity
- Catalase activity
- Ascorbate peroxidase activity
- Glutathione reductase activity
- Reduced glutathione content
- Reduced ascorbate content

3.7.5.1 Activity of Antioxidant Enzymes

3.7.5.1.1 Enzyme Extraction

Fresh leaf tissue (200 mg) was homogenized with an extraction buffer containing 100 mM K-phosphate buffer (pH 7.0), 0.5% Triton X-100 and 1% PVP using chilled mortar and pestle. The homogenate was centrifuged at 15,000×g for 20 min at 4°C. The supernatant obtained was used for the enzymatic assays. For ascorbate peroxidase, extraction buffer was supplemented with 2 mM ascorbate. Enzyme extract was used for the assay of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase.

3.7.5.1.1.1 Superoxide Dismutase

The method described by Dhindsa *et al.* (1981) was followed with slight modification for the estimation of superoxide dismutase activity.

3.7.5.1.1.1.1 Enzyme Assay

Superoxide dismutase activity in the supernatant was assayed by its ability to inhibit the photochemical reduction. The assay mixture consisting of 1.5 ml of 0.1 M K-phosphate buffer (pH 7.8), 0.2 ml of methionine, 0.1 ml enzyme extract with equal amount of 1 M $NaCO_3$, 2.25 mM NBT solution, 3 mM EDTA, 60 µM riboflavin and 1.0 ml of de-ionized water, was taken in test tubes which were incubated under light of 15 W inflorescent lamp for 10 min at 25°C. Blank 'A' containing all the above substances of the reaction mixture, along with the enzyme extract were placed in the dark. Blank 'B' containing all the above substances of reaction mixture except the enzyme was placed in light along with the sample. The reaction was terminated by switching off the light, and the tubes were covered with black cloth. The non-irradiated reaction mixture containing enzyme extract did not develop light blue colour. Absorbance of the samples along with Blank 'B' was read at 560 nm against the Blank 'A'. The difference of percent reduction in colour between Blank 'B' and the sample was then calculated.

A reduction of 50% in the colour was considered as one Unit of the enzyme activity.

3.7.5.1.1.2 Catalase

Catalase activity in leaves was determined by the method of Aebi (1984) with slight modification.

3.7.5.1.1.2.1 Enzyme Assay

Catalase activity was determined by monitoring the disappearance of H_2O_2 by measuring the absorbance at 240 nm. Reaction was carried out in a final volume of 2.0 ml of reaction mixture containing 0.5 M K-phosphate buffer (pH 7.2) with 0.1 ml 3 mM EDTA, 0.1 ml of enzyme extract and 0.1 ml of 3 mM H_2O_2. The reaction was allowed to run for 5 min. The activity of the enzyme was calculated by using the extinction coefficient 0.036 mM^{-1} cm^{-1}. One Unit of the enzyme activity is defined as the amount necessary to decompose 1 µmol of H_2O_2 per min at 25°C.

3.7.5.1.1.3 Ascorbate Peroxidase

Ascorbate peroxidase activity was determined by the method of Nakano and Asada (1981).

3.7.5.1.1.3.1 Enzyme Assay

Ascorbate peroxidase activity was determined by the decrease in the absorbance of ascorbate at 290 nm due to its enzymatic breakdown. The volume of 1.0 ml of 50 mM K-phosphate buffer (pH 7.2) contained 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and 0.1 ml enzyme extract. The reaction was allowed to run for 5 min at 25°C. Ascorbate peroxidase activity was calculated by using the extinction coefficient 2.8 mM^{-1} cm^{-1}. One Unit of enzyme activity is defined as the amount necessary to decompose 1 µmol of substrate consumed per min at 25°C.

3.7.5.1.1.4 Glutathione Reductase

Glutathione reductase activity was determined by the method of Foyer and Halliwell (1976) and modified by Rao (1992).

3.7.5.1.1.4.1 Enzyme Assay

Glutathione reductase activity was determined by monitoring the glutathione-dependent oxidation of NADPH at its absorption maxima of wavelength 340 nm. Reaction mixture (1.0 ml) contained 0.2 mM NADPH, 0.5 mM GSSG and 50 µl of the enzyme extract. The reaction was allowed to run for 5 min at 25°C. Corrections were made for any GSSG oxidation in the absence of NADPH. The activity of the enzyme

was calculated by using the extinction coefficient (6.2 mM^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount necessary to decompose 1μmol of NADPH per min at 25°C.

3.7.5.1.2 Glutathione

Reduced glutathione (GSH) content was determined by the glutathione recycling method of Anderson (1985). The details are as follows:

3.7.5.1.2.1 Procedure

Fresh leaf tissue (200 mg) was homogenized in 2 ml of 5% sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000×g for 10min. To 0.5 ml of supernatant, 0.6 ml of 100 mM phosphate buffer (pH 7.0) and 40 μl of 5', 5'-dithiobis-2-nitrobenzoic acid (DTNB) were added. After 2 min the absorbance was read at 412 nm. Standard curve for calculations was prepared from GSH covering a range of 10-100 nmol.

3.7.5.1.3 Ascorbate

Estimation of ascorbic acid (AsA) content was done by the method of Law *et al.* (1983) with slight modification.

3.7.5.1.3.1 Procedure

Fresh leaf tissue (200 mg) was ground in 2 ml of 100 mM K-phosphate buffer (pH 7.0) with 1 mM EDTA and centrifuged at 10,000×g for 10 min. The supernatant was collected and 200 μl of 10% TCA was added. After mixing the solution by vortex, it was allowed to stand for 5 min. Following mixing, 10 μl of 5 M NaOH was added and centrifuged for 2 min. To 200 μl of supernatant, 200 μl of 150 mM K-phosphate buffer (pH 7.4), 100 μl of 10 mM 5',5'-dithiobis-2-nitrobenzoic acid were added, mixed thoroughly and left at room temperature for 15 min. After mixing 100 μl of 0.5% N'N-ethylemaleimide (NEM) was added. Samples were mixed by vortex and incubated at 24°C for 30 sec. To each sample 400 μl of 10% TCA, 400 μl H_3PO_4, 400 μl of 4% bipyridyl dye (N'N-dimethyl bipyridyl) and 200 μl of 3% $FeCl_3$ were added. After vortex mixing, samples were incubated at 37°C for 1 h and the absorbance was recorded at 525 nm. A standard curve in the range of 10-100 nmol of ascorbic acid was used for calibration. Values in both cases were corrected for the absorbance eliminating the supernatant in the blank prepared separately for AsA and AsA+DHAs.

3.7.6 Leaf Protein Content

Protein content was estimated by the method of Lowry *et al.* (1951). Plant material was ground to fine powder in a mortar and pestle. Five hundred mg of sample was further ground in 5 ml of 5% trichloroacetic acid solution. From this, 0.1 ml sample was taken in test tube and the volume was made to 1 ml with de-ionized water. Five ml of reagent C was added to the test tube and centrifuged at 4,000 rpm. Then, 0.5 ml of Reagent D was added to the test tube and mixed well. The mixture was incubated at room temperature for 30 min in the dark for maximum colour development. The intensity of the blue colour developed was read at 660 nm.

1. Reagent C: Prepared by mixing 50 ml of Reagent A (2% sodium carbonate and 0.1 N NaOH in 1:1 ratio) and 1 ml of reagent B (0.5% copper sulphate and 1% potassium sodium tartarate in 1:1 ratio).
2. Reagent D: Prepared by mixing 50 ml of 2% sodium carbonate solution in 1 ml of Reagent B.

3.7.6.1 Standard Curve

50 mg of Bovine serum albumin was dissolved in de-ionized water in a 50 ml volumetric flask and the volume was maintained. From this solution, 10 ml was taken and diluted to 50 ml in another 50 ml volumetric flask. One ml of this solution contained 200 µg protein. Different concentrations such as 0.2, 0.4, 0.6, 0.8 and 1.0 ml from this solution were taken to different test tubes and the volume was maintained to 1 ml. To this, 5 ml of Reagent C was added, mixed well and allowed to stand for 10 min followed by the addition of 0.5 ml of Reagent D and incubated at room temperature in the dark for 30 min for maximum colour development. The colour intensity was read at 660 nm.

Standard curve was plotted using different concentrations of the working standard versus optical density. With the help of this standard curve the amount of protein present in the samples was calculated.

3.7.7 Growth Characteristics

- Leaf area per plant
- Dry mass per plant
- Relative growth rate

Plants were uprooted carefully from the pots, washed to remove dust. Leaf area was measured with a leaf area meter (LA 211, Systronics, New Delhi, India). Dry mass was recorded after drying the sample in a hot air oven at 80°C till constant weight. The dried material was weighed on an electronic balance (CY204, Scalteo Ins., Germany).

Relative growth (RGR) was calculated using the following formula given by Radford (1967).

$$RGR = [\ln(W_2) - \ln(W_1)]/(t_2 - t_1)$$

where W_1 and W_2 are plant dry mass at times t_1 and t_2.

3.7.8 Yield Characteristics

Yield is the final manifestation of morphological, physiological and biochemical traits of a crop.

At harvest (120 DAS) following parameters were recorded.

- Number of pods per plant
- Number of seeds per pod
- Seed yield per plant

At harvest, pods were collected and counted. The number of seeds from each pod was counted. From the produce of the pot, a sample of thousand seeds was randomly drawn and the weight was recorded using electronic balance. The total seeds from a plant in each treatment were cleared, sun-dried and weighed to compute seed yield per plant.

3.8 Statistical Analysis

Data were analyzed statistically using the Statistical Package for the Soil Sciences (SPSS, 10.0 for Windows). Standard error was calculated and analysis of variance was performed on the data to determine least significant difference (LSD) for significant data to identify difference in the mean of the treatment. The treatment means were separated using LSD test. Different letters indicate significant difference at $P < 0.05$. Regression analysis to establish correlation between ATP-sulfurylase activity and photosynthesis or shoot dry mass was done in Experiment 1.

Experimental Results

Contents

EXPERIMENTAL RESULTS

The chapter 'Experimental Results' presents herein mainly the changes in photosynthetic characteristics, water relations, content of nutrients and ions, oxidative stress, various components of antioxidant defense system, growth and yield characteristics of mustard cultivars.

4.1 Experiment 1: Selection of cultivars differing in ATP-sulfurylase activity and S accumulation capacity/ S-efficiency

Experiment was conducted to select the S-efficient and S-inefficient mustard cultivars by studying the activity of ATP-sulfurylase and S content of four mustard cultivars, namely, Alankar, Varuna, Pusa Jai Kisan and SS2. The activity of leaf ATP-sulfurylase, content of SO_4^{2-} and N in leaf, photosynthesis and growth were measured at 30 and 60 DAS. As mentioned in Material and Methods section root SO_4^{2-} content was determined at 30 DAS and S accumulation capacity was determined as sulfate transport index (STI: calculated as the ratio of sulfate content in root and leaf and expressed as percentage). The relationship of ATP sulfurylase activity with photosynthesis and shoot dry mass of the four cultivars was worked out. Results are described below in detail (Table 3).

4.1.1 ATP-sulfurylase activity and S content

The cultivars differed in ATP-sulfurylase activity and the pattern of ATP-sulfurylase activity in the cultivars was Pusa Jai Kisan > Alankar > Varuna > SS2.

ATP-sulfurylase activity increased from 30 to 60 DAS in all the cultivars, but in Pusa Jai Kisan, the activity was increased by 16.7% to 25.8% from 30 to 60 DAS. However, leaf sulfate content was greatest in Alankar followed by Pusa Jai Kisan, Varuna and SS2 at both the sampling times. All the cultivars differed in root sulfate content and in sulfate transport index. Pusa Jai Kisan showed maximum STI followed by Alankar, Varuna and SS2.

4.1.2 Net Photosynthetic rate

Net photosynthetic rate increased in all the cultivars from 30 to 60 DAS. Pusa Jai Kisan gave the maximum photosynthetic rate followed by Alankar and Varuna, which gave equal rate of photosynthesis. SS2 gave the lowest photosynthesis value at both the stages.

Table 3. Contents of root and leaf sulfate (SO_4^{2-}) and leaf nitrogen (N) (mg kg^{-1} dry mass), sulfate transport index (STI) (%), activity of ATP-sulfurylase (μmol g^{-1} protein s^{-1}), net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), leaf area (cm^2 per plant), and shoot dry mass (g per plant) of four cultivars of mustard (*Brassica juncea* L.) at 30 or 60 d after sowing (DAS). Mean ±SE (n = 3).
*Values are significantly different at $p<0.05$. STI was calculated as the ratio of root to leaf sulfate content.

	DAS	Alankar	Varuna	Pusa Jai Kisan	SS2
ATP-sulfurylase activity	30	2.77±0.11*	2.55±0.08	3.61±0.12	1.67±0.09
	60	3.39±0.10*	3.00±0.09	4.55±0.14	2.55±0.11
Leaf SO_4^{2-} content	30	5.21±0.40*	3.46±0.30	4.32±0.40	3.02±0.20
	60	9.11±0.50*	5.36±0.20	7.97±0.30	4.53±0.30
Root SO_4^{2-} content	30	2.20±0.06	1.22±0.03	2.65±0.07*	1.02±0.02
Sulfate transport index	30	42.23±2.66	35.26±2.15	61.34±3.81*	33.77±1.56
Leaf N content	30	58.38±2.40*	56.98±1.80	64.54±1.80	54.60±2.10
	60	60.55±2.10*	58.36±2.00	67.48±2.20	55.70±1.80
Net photosynthetic rate	30	13.25±0.56*	12.85±0.42	14.56±0.50	10.00±0.42
	60	24.82±7.40*	23.23±7.80	28.22±8.20	18.07±7.50
Leaf area	30	158.0±9.4*	150.0±9.2	150.0±9.2	140.0±7.4
	60	188.0±10.5*	175.0±6.6	230.0±12.5	152.0±6.1
Shoot dry mass	30	7.33±0.32*	5.43±0.18	9.17±0.32	4.20±0.22
	60	12.10±0.72*	8.21±0.62	16.88±0.82	6.80±0.32

4.1.3 Nitrogen content

Among cultivars, Pusa Jai Kisan, followed by Alankar gave maximum leaf N content at the both stages. The order of the cultivars for nitrogen content was Pusa Jai Kisan > Alankar >Varuna > SS2.

4.1.4 Growth characteristics

All the cultivars differed in growth characteristics and the pattern of growth in the cultivars was Pusa Jai Kisan > Alankar > Varuna > SS2. Maximum leaf area and shoot dry mass was recorded for Pusa Jai Kisan and minimum for SS2.

All the cultivars exhibited strong relationship of ATP-sulfurylase activity with photosynthesis and shoot dry mass (Figure 3).

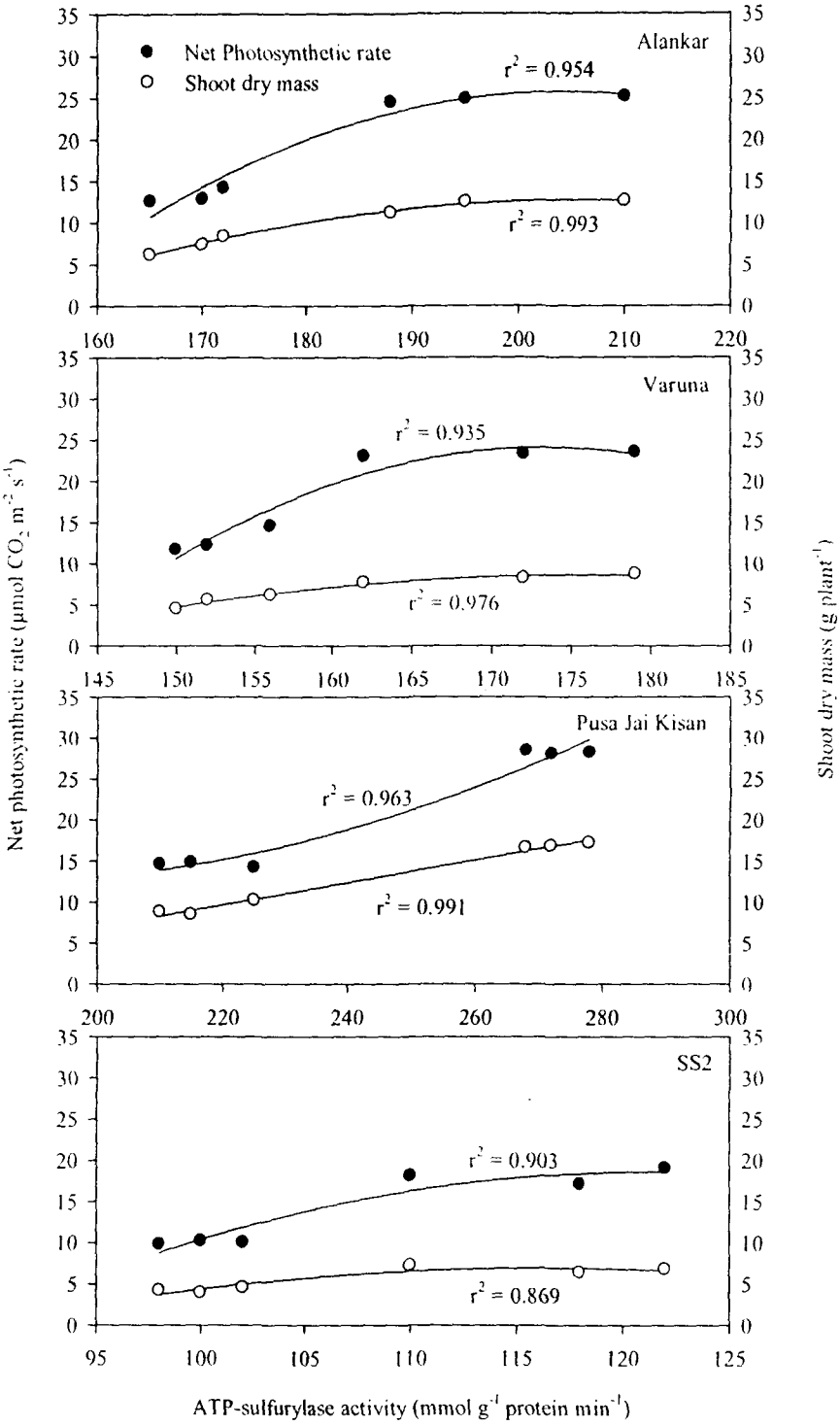


Figure 3. Relationship of ATP-sulurylase activity with photosynthesis, shoot dry mass of four mustard (*Brassica juncea* L.) cultivars (published as Nazar *et al.* 2008).

4.1.5 Summary of Experiment 1

- The cultivar Pusa Jai Kisan exhibited maximum ATP-sulfurylase activity, photosynthesis and plant growth.
- A higher ATP-sulfurylase activity and sulfate transport index in Pusa Jai Kisan indicates its higher sulfate accumulation capacity.

- A strong positive correlation ($P<0.01$) between ATP-sulfurylase activity and photosynthesis and shoot dry mass was found.
- On the basis of overall performance of the cultivars, Pusa Jai Kisan emerged as sulfur-efficient and SS2 as sulfur-inefficient types.

4.2 Experiment 2: Study on the effect of salinity stress on mustard types differing in ATP-sulfurylase activity/ S-accumulation capacity

Experiment 2 was conducted on the basis of findings of Experiment 1. As observed in Experiment 1, Pusa Jai Kisan emerged as S-efficient and SS2 as S-inefficient mustard cultivars. Thus, another experiment was conducted with the aim of studying the influence of 0, 50 and 100 mM NaCl on sulfur assimilation, photosynthetic traits, water relations, content of nutrients and ions, oxidative stress, various components of antioxidant defense system and growth in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (low ATP-sulfurylase activity) cultivars of mustard at 30 and 60 DAS and yield characteristics at 120 DAS. Following sections present the results in details (Figures 4-36).

4.2.1 ATP-sulfurylase activity and S accumulation

Salinity stress increased ATP-sulfurylase activity and S content, and was significantly higher than control in both the cultivars. Pusa Jai Kisan exhibited higher ATP-sulfurylase activity and S content than SS2 at both the NaCl levels (50 and 100 mM NaCl). However, the effect of 50 and 100 mM NaCl on ATP-sulfurylase activity and S content did not differ significantly in both the cultivars (Figures 4-5).

In Pusa Jai Kisan, ATP-sulfurylase activity was increased by 36.7% and 35.3%, S content by 32.7% and 31.2% due to 50 and 100 mM NaCl, respectively in comparison to control at 30 DAS. At later growth stage (60 DAS), the activity of ATP-sulfurylase increased by 40.0% and 38.9%, S content by 21.3% and 19.3% due to 50 and 100 mM NaCl, respectively compared to control (Figures 4-5). In SS2, the ATP-sulfurylase activity was increased by 18.6% and 17.4%, S content by 16.1% and 15.4% due to 50 and 100 mM NaCl in comparison to control at 30 DAS. At 60 DAS the activity was increased by 21.3% and 19.3%, S content by 20.0% and 18.7% due to 50 and 100 mM NaCl compared to control.

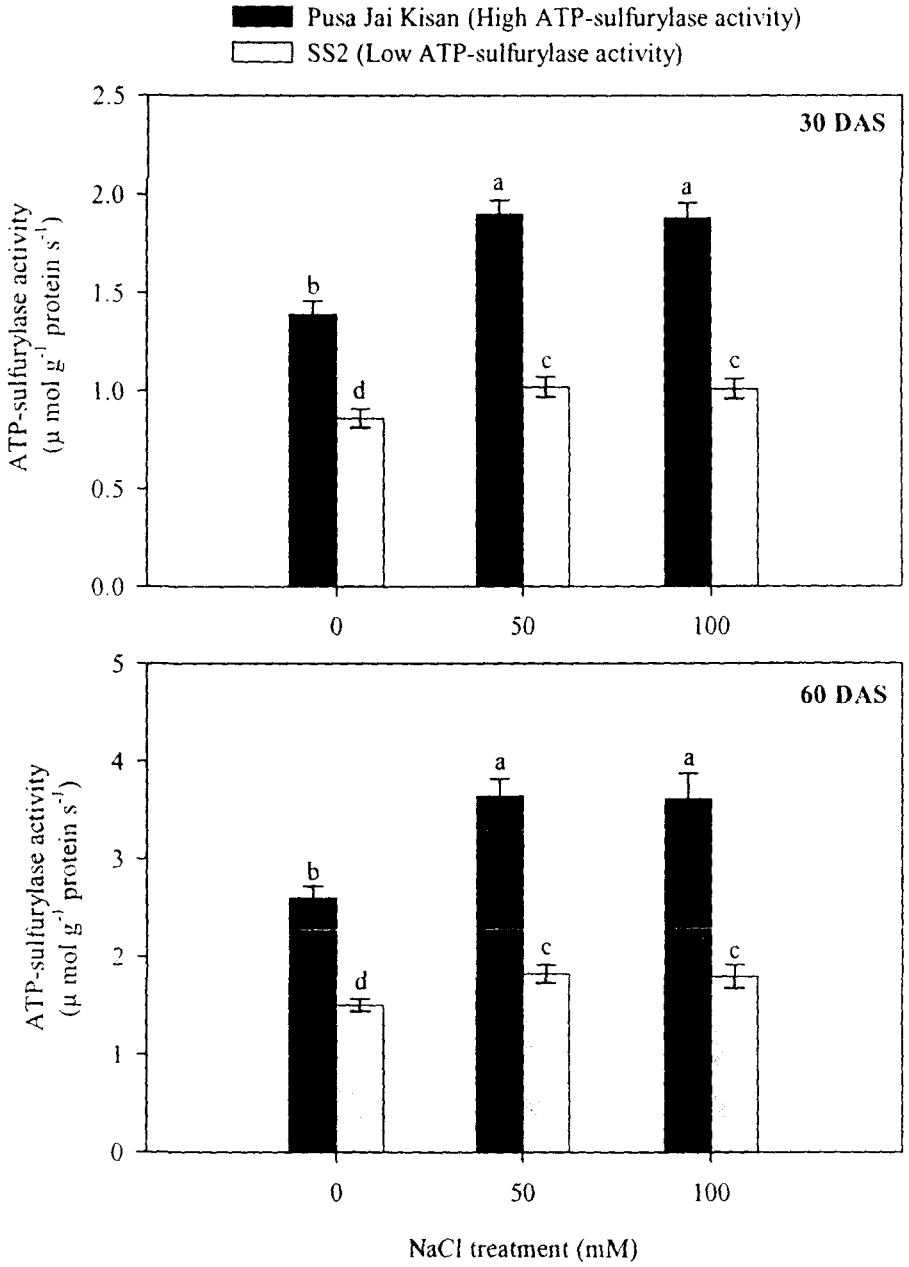


Figure 4. Effect of NaCl treatments on ATP-sulfurylase activity of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

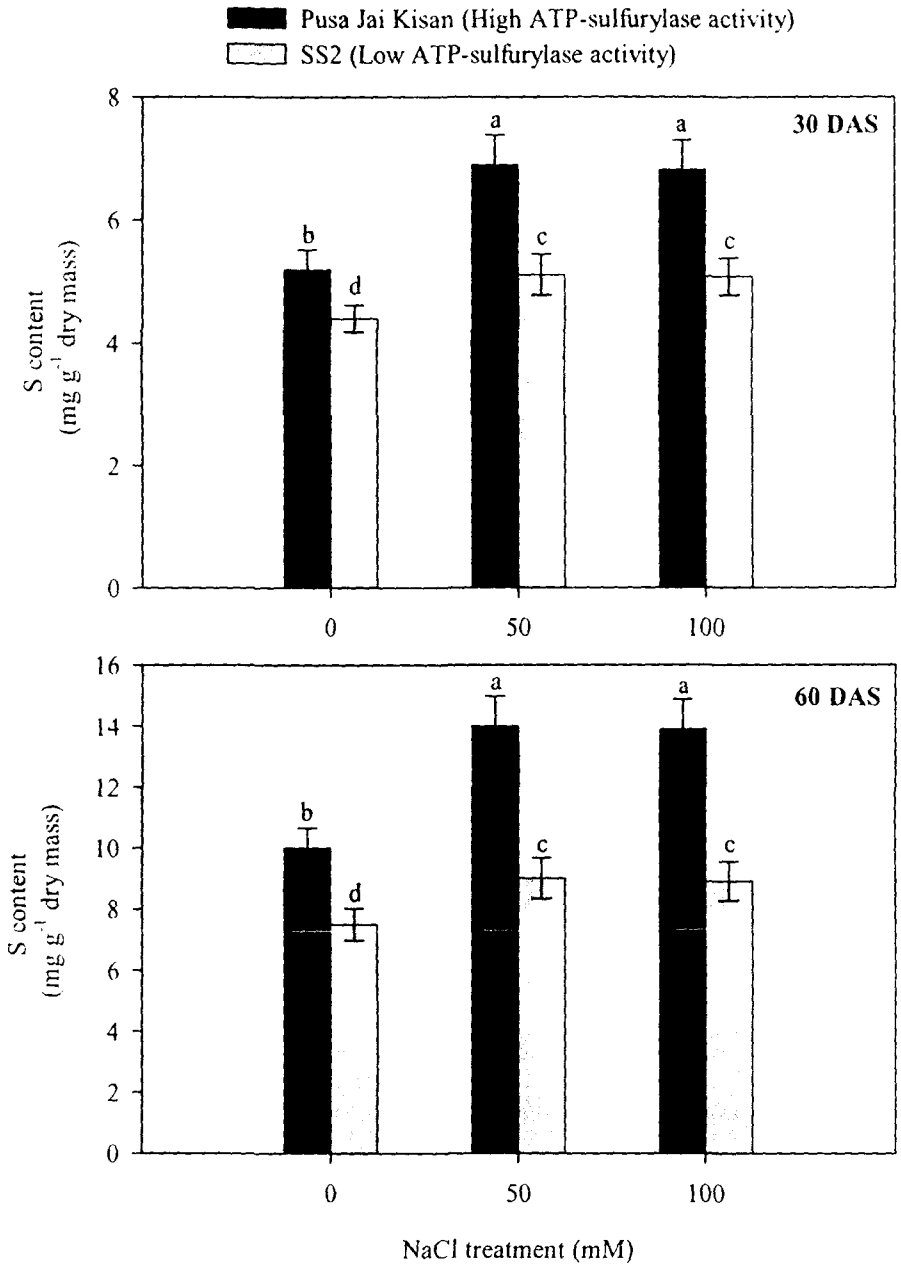


Figure 5. Effect of NaCl treatments on S content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

4.2.2 Photosynthetic characteristics

Higher significant reduction in photosynthetic characteristics (net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, water-use efficiency and chlorophyll fluorescence) was noted with NaCl levels, and the effect of 100 mM NaCl was more conspicuous in SS2, at both the sampling times than Pusa Jai Kisan (Figures 6-11).

Photosynthetic rate, stomatal conductance and intercellular CO_2 concentration decreased maximally in SS2 with 100 mM NaCl in comparison to control. In Pusa Jai Kisan the decrease in net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration was 19.3%, 11.9% and 12.3%, respectively due to 100 mM NaCl at 30 DAS in comparison to control. At 60 DAS, the decrease in these characteristics was 29.5%, 22.2% and 21.4%, respectively due to 100 mM NaCl in comparison to control.

The decrease in net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration in SS2 was 39.9%, 16.6% and 22.0% at 30 DAS, and 48.7%, 33.3% and 33.1%, respectively due to 100 mM NaCl at 60 DAS in comparison to control.

Transpiration rate increased with NaCl treatment and the increase was higher in SS2 compared to Pusa Jai Kisan. However, the effect of 50 and 100 mM NaCl on transpiration rate did not differ significantly in both the cultivars at 30 DAS. The transpiration rate in Pusa Jai Kisan was increased by 41.0% and 55.3% due to 100 mM NaCl at 30 and 60 DAS, respectively, in comparison to control.

A higher increase in transpiration rate in SS2 of 49.3% and 73.3% due to 100 mM NaCl at 30 and 60 DAS, respectively was noted in comparison to control.

Water-use efficiency and chlorophyll fluorescence decreased significantly and maximally in plants treated with 100 mM NaCl in comparison to control. Water-use efficiency and chlorophyll fluorescence in Pusa Jai Kisan were decreased by 42.7% and 22.2% at 30 DAS and 51.2% and 34.6% at 60 DAS due to 100 mM NaCl compared to their respective control. In SS2, water-use efficiency and chlorophyll fluorescence were decreased by 59.8% and 46.5% at 30 DAS and 70.4% and 38.9% at 60 DAS with 100 mM NaCl compared to control.

4.2.3 Water Relations

Leaf water potential and osmotic potential decreased due to salinity stress and were found significant with the increase in salinity levels at both sampling times (Figures 12-13).

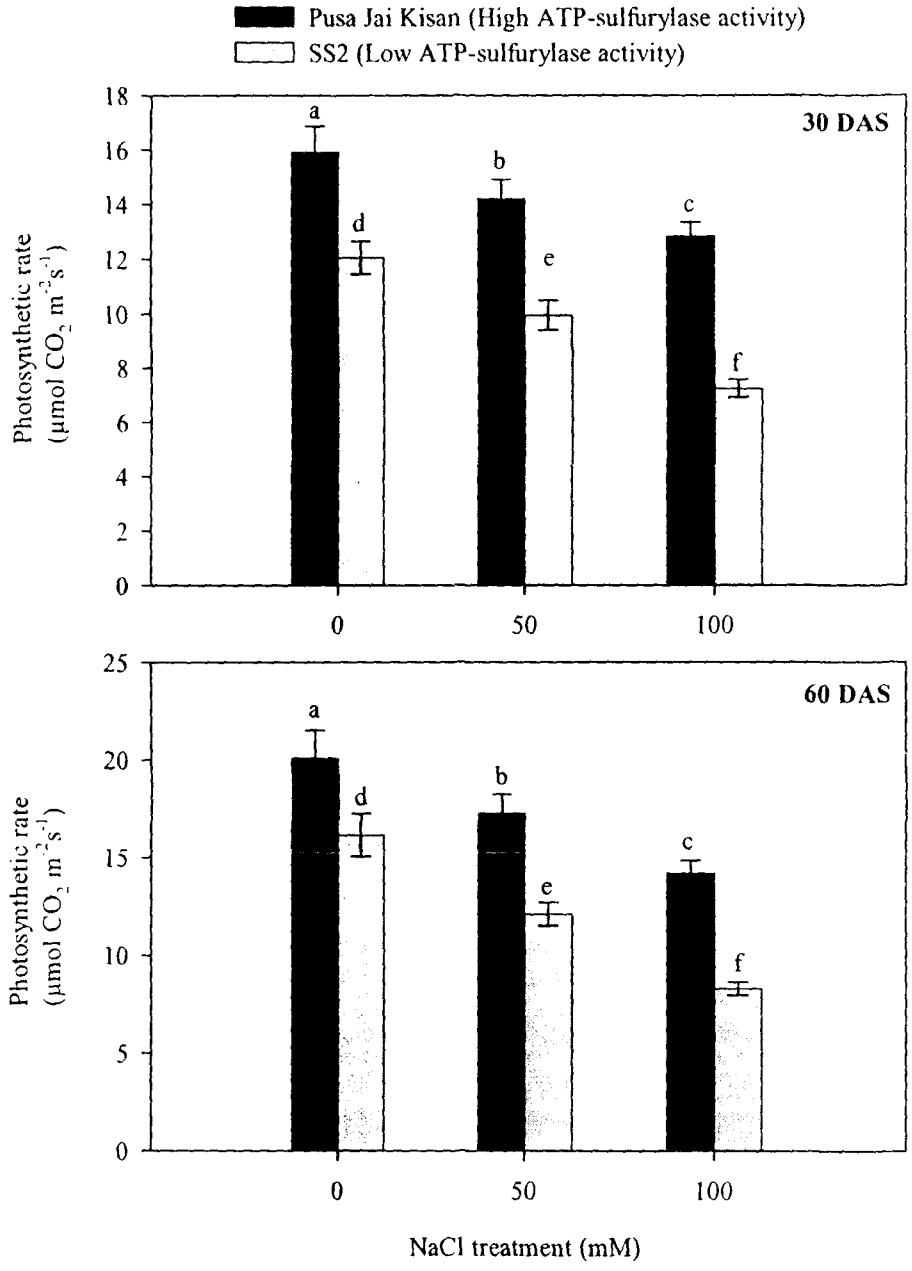


Figure 6. Effect of NaCl treatments on photosynthetic rate of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

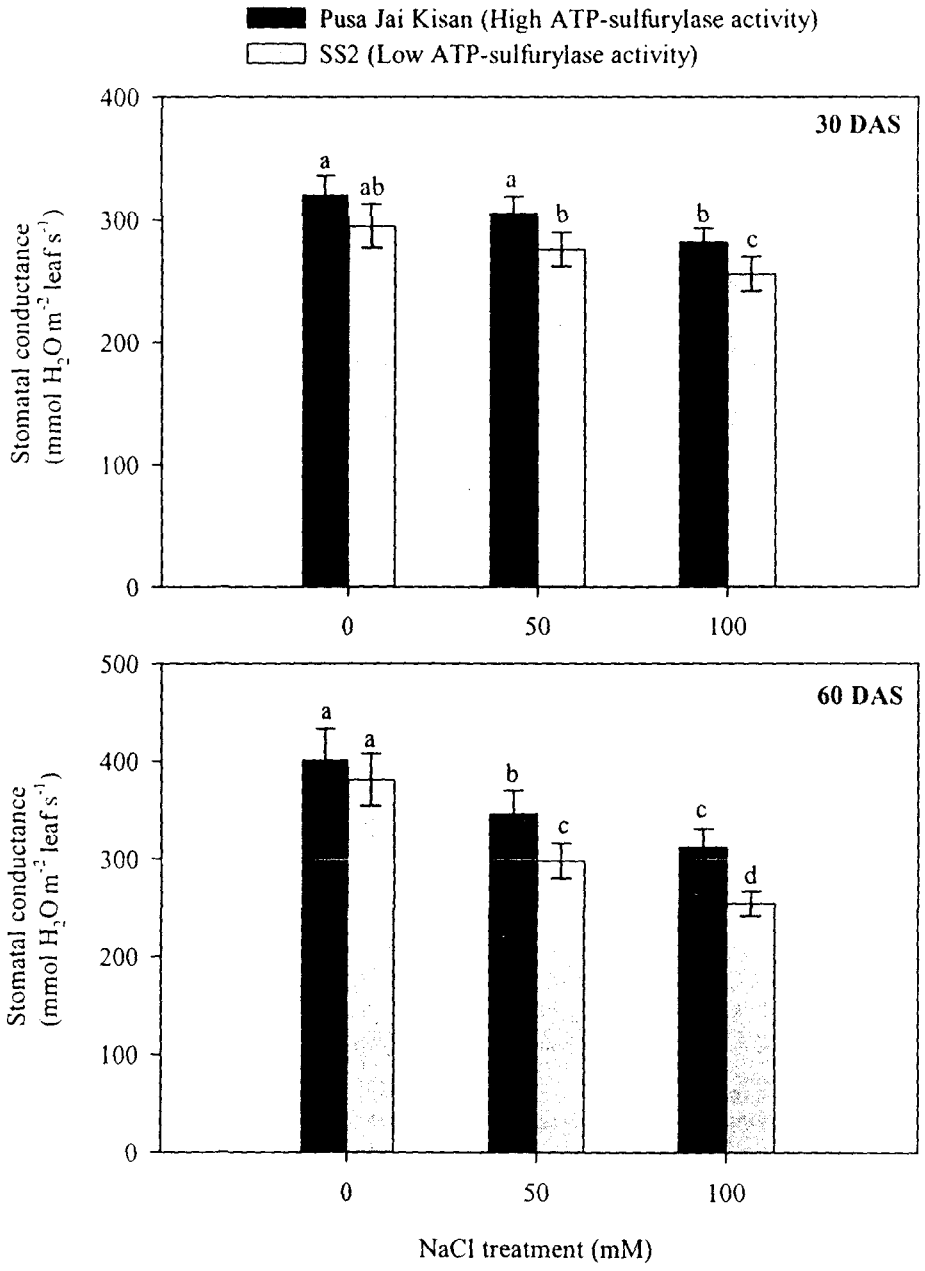


Figure 7. Effect of NaCl treatments on stomatal conductance of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

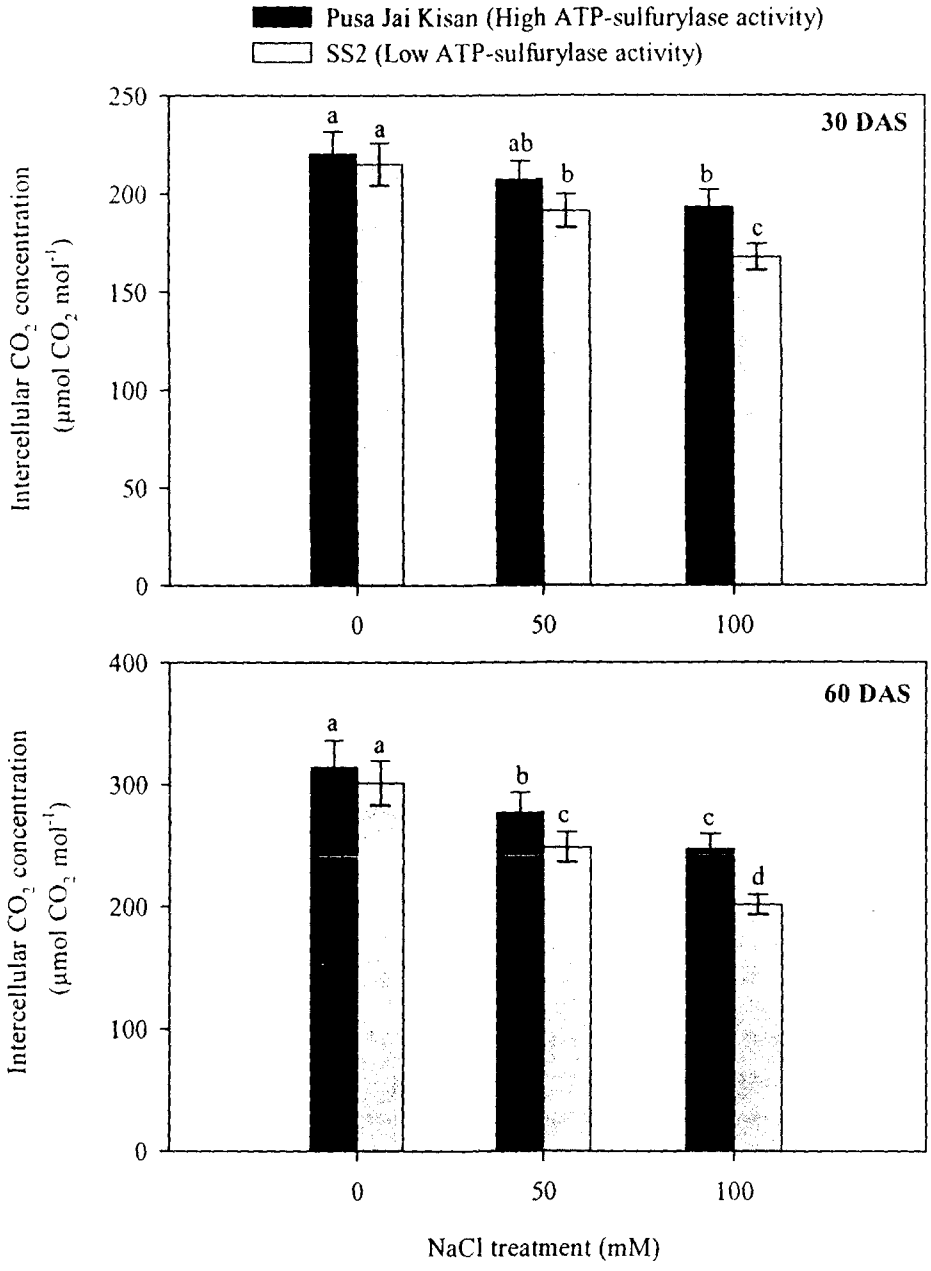


Figure 8. Effect of NaCl treatments on intercellular CO_2 concentration of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

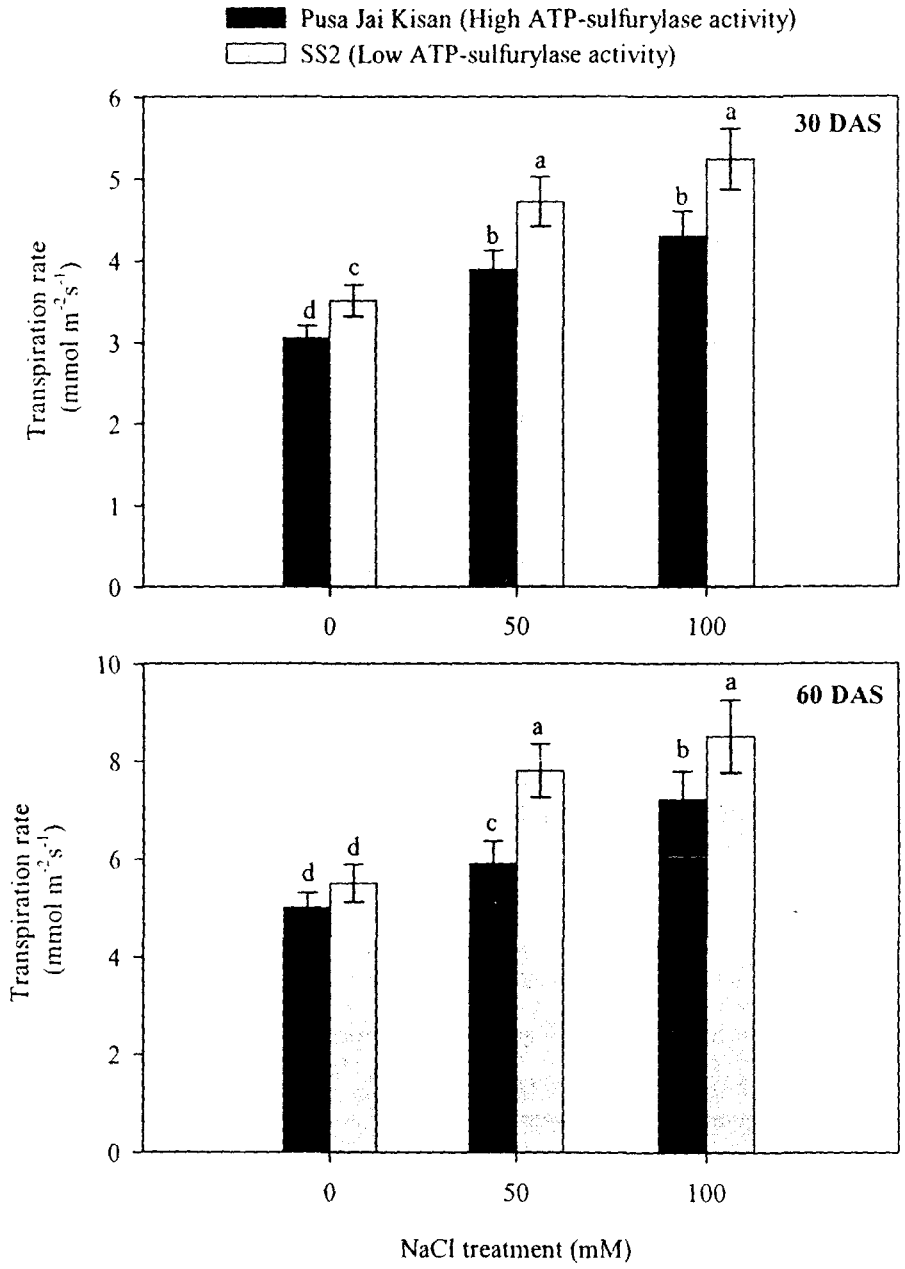


Figure 9. Effect of NaCl treatments on transpiration rate of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

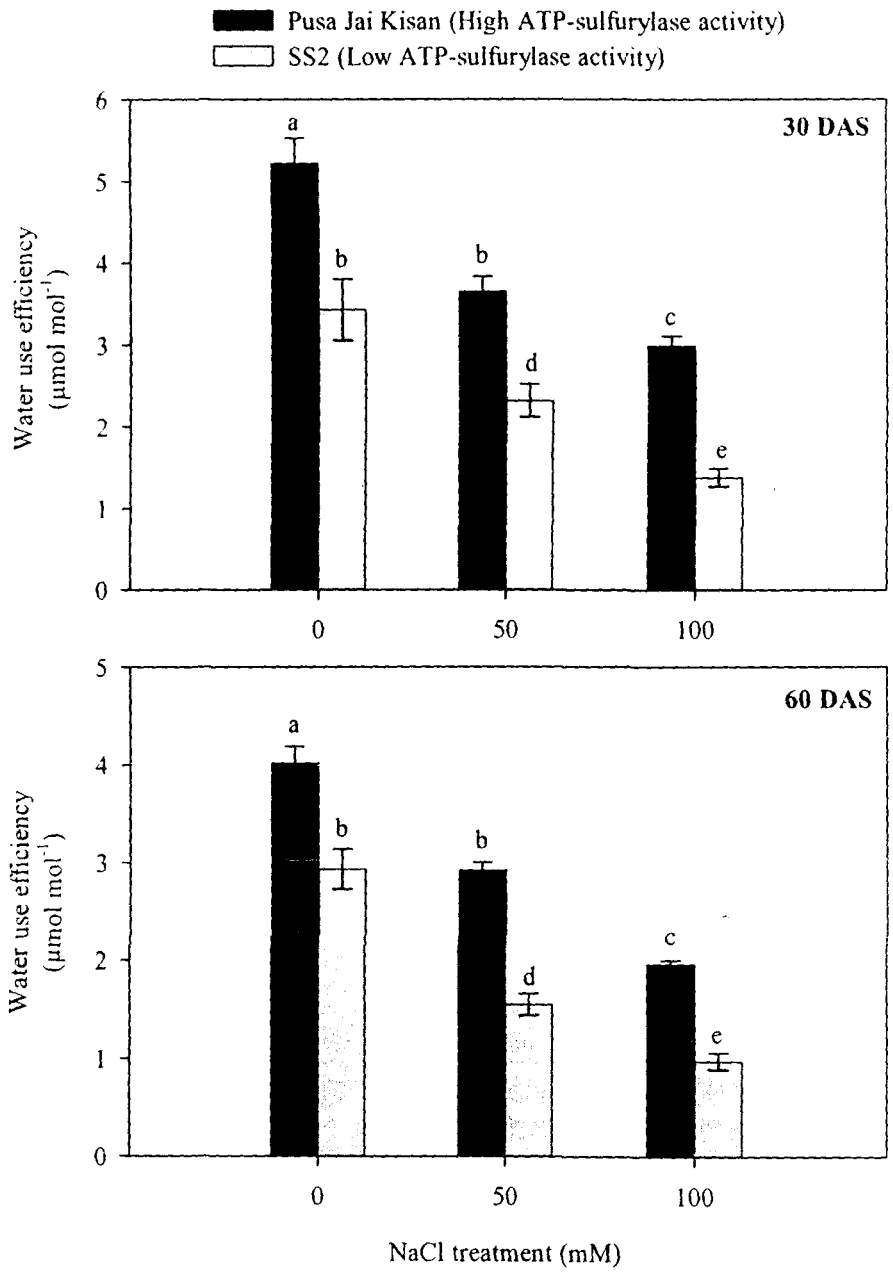


Figure 10. Effect of NaCl treatments on water use efficiency of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

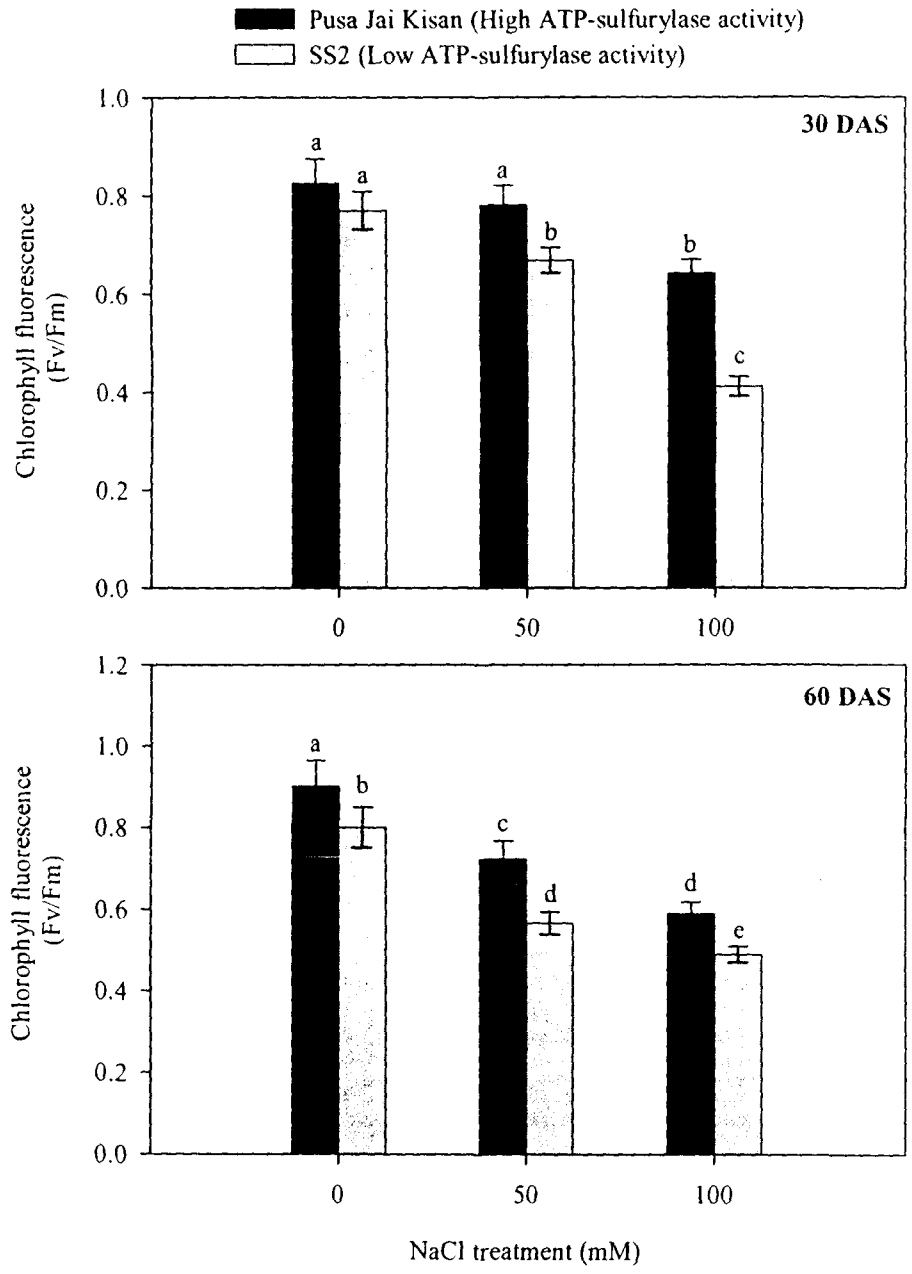


Figure 11. Effect of NaCl treatments on chlorophyll fluorescence of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

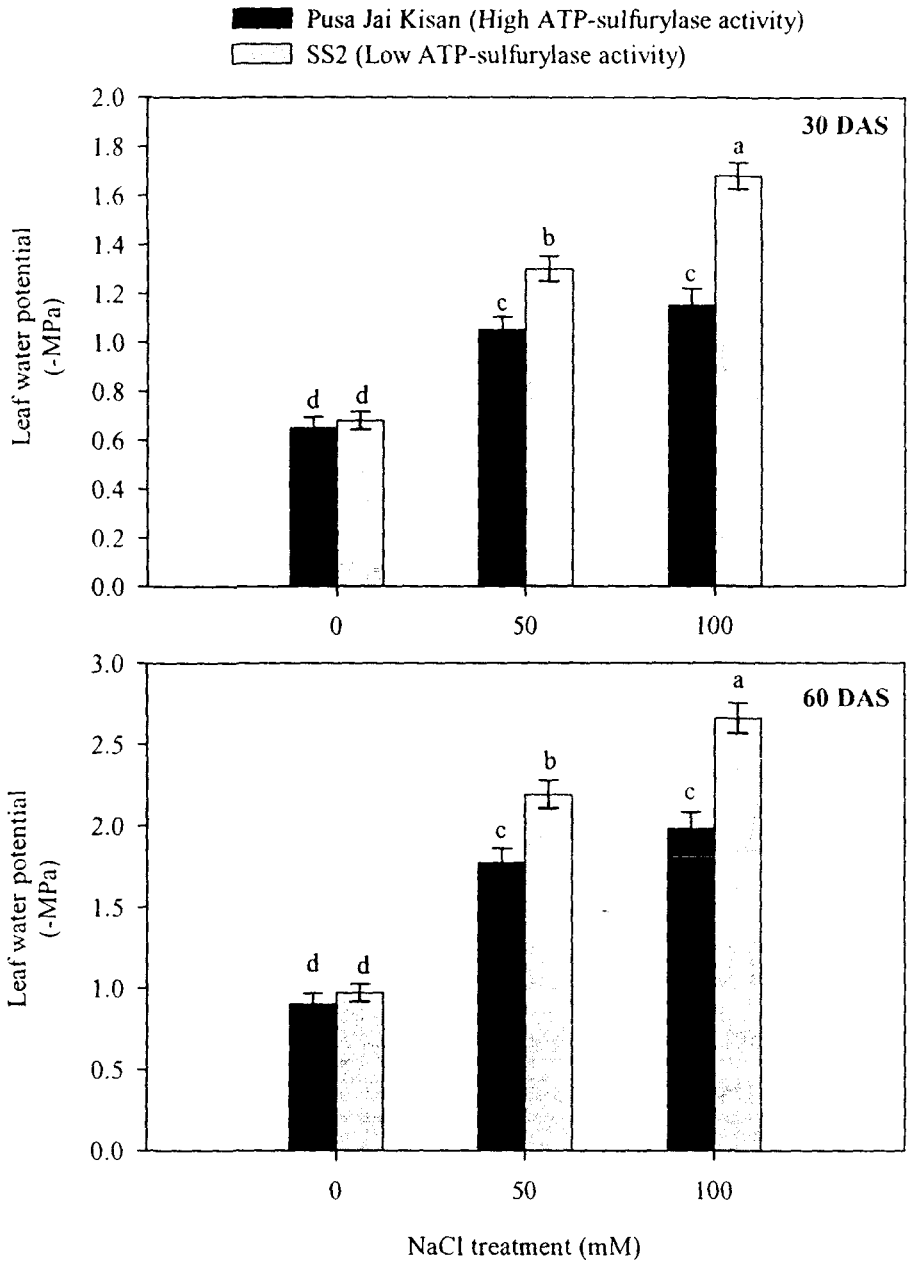


Figure 12. Effect of NaCl treatments on leaf water potential of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

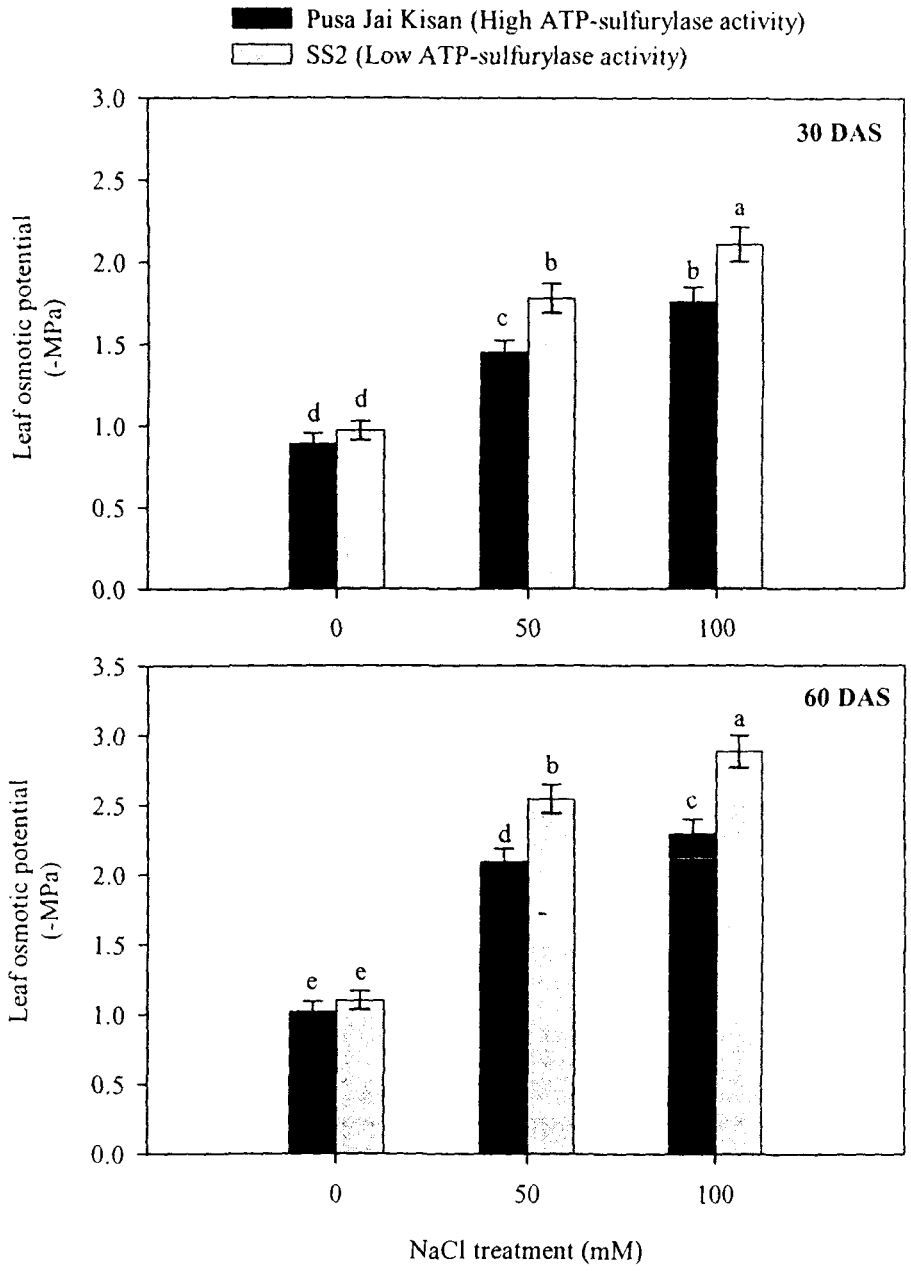


Figure 13. Effect of NaCl treatments on leaf osmotic potential of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

The treatment of 100 mM NaCl decreased leaf water potential and osmotic potential in Pusa Jai Kisan and SS2. The decreases in these characteristics due to 100 mM NaCl were 77.05 and 97.6% at 30 DAS, 120.0% and 124.5% at 60 DAS in Pusa Jai Kisan in comparison to the respective control. A higher decrease in the above characteristics of 154.5% and 117.5% at 30 DAS, 174.2% and 161.8% at 60 DAS in SS2 with 100 mM NaCl was noted in comparison to the respective control.

4.2.4 Content of nutrients and ions

4.2.4.1 Nutrients

Salinity stress significantly reduced the content of leaf N, P, K and Ca in both high ATP-sulfurylase activity (Pusa Jai Kisan) and low ATP-sulfurylase activity (SS2) cultivars but the reduction was more prominent in SS2 than Pusa Jai Kisan with 100 mM NaCl (Figures 14-17).

The decrease in leaf N, P, K and Ca content in Pusa Jai Kisan with 100 mM NaCl was 28.2%, 33.6%, 18.2% and 22.7% at 30 DAS, and 31.5%, 46.1%, 18.4% and 28.1%, respectively at 60 DAS compared to control. The cultivar SS2 showed a higher decrease of 39.1%, 46.0%, 24.15 and 35.2% at 30 DAS and 46.9%, 50.3%, 29.9% and 46.1% at 60 DAS, respectively with 100 mM NaCl compared to control.

4.2.4.2 Ions

In both the cultivars, Na+ content in root and leaf was higher in NaCl treatments than control. Both the cultivars accumulated equally Na+ in root under 50 and 100 mM NaCl. However, the content of Na+ in leaf was significantly higher in SS2 than Pusa Jai Kisan at both the NaCl levels (Figures 18-21).

Root and leaf Na+ in Pusa Jai Kisan following treatment of 100 mM NaCl increased by 31.5% and 32.9% at 30 DAS, 29.3% and 35.5% at 60 DAS in comparison to control. In SS2, the content was increased by 58.5% and 32.7% at 30 DAS, 30.6% and 47.1% at 60 DAS with 100 mM NaCl in comparison to control.

Salinity stress increased Cl- content in root maximally with 100 mM NaCl in both the cultivars. At both the NaCl levels the content of Cl- in root was higher in Pusa Jai Kisan than SS2, whereas leaf Cl- content was higher in SS2. The treatment of 100 mM NaCl did not significantly enhance leaf Cl- content in both the cultivars compared to 50 mM NaCl.

In Pusa Jai Kisan the content of root and leaf Cl^- was increased by 32.7% and 19.7% at 30 DAS, 40.0% and 24.8% at 60 DAS due to 100 mM NaCl in comparison to

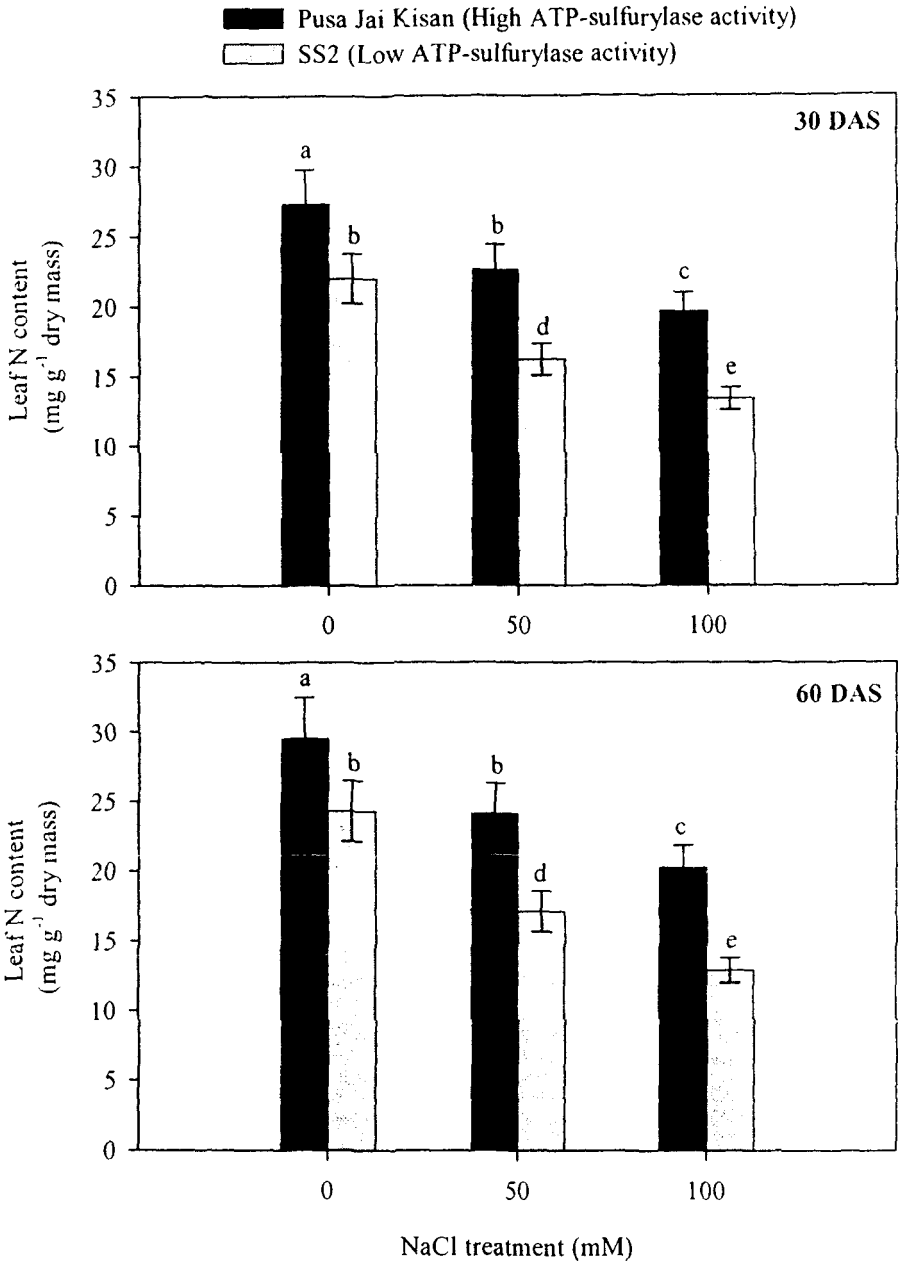


Figure 14. Effect of NaCl treatments on leaf N content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

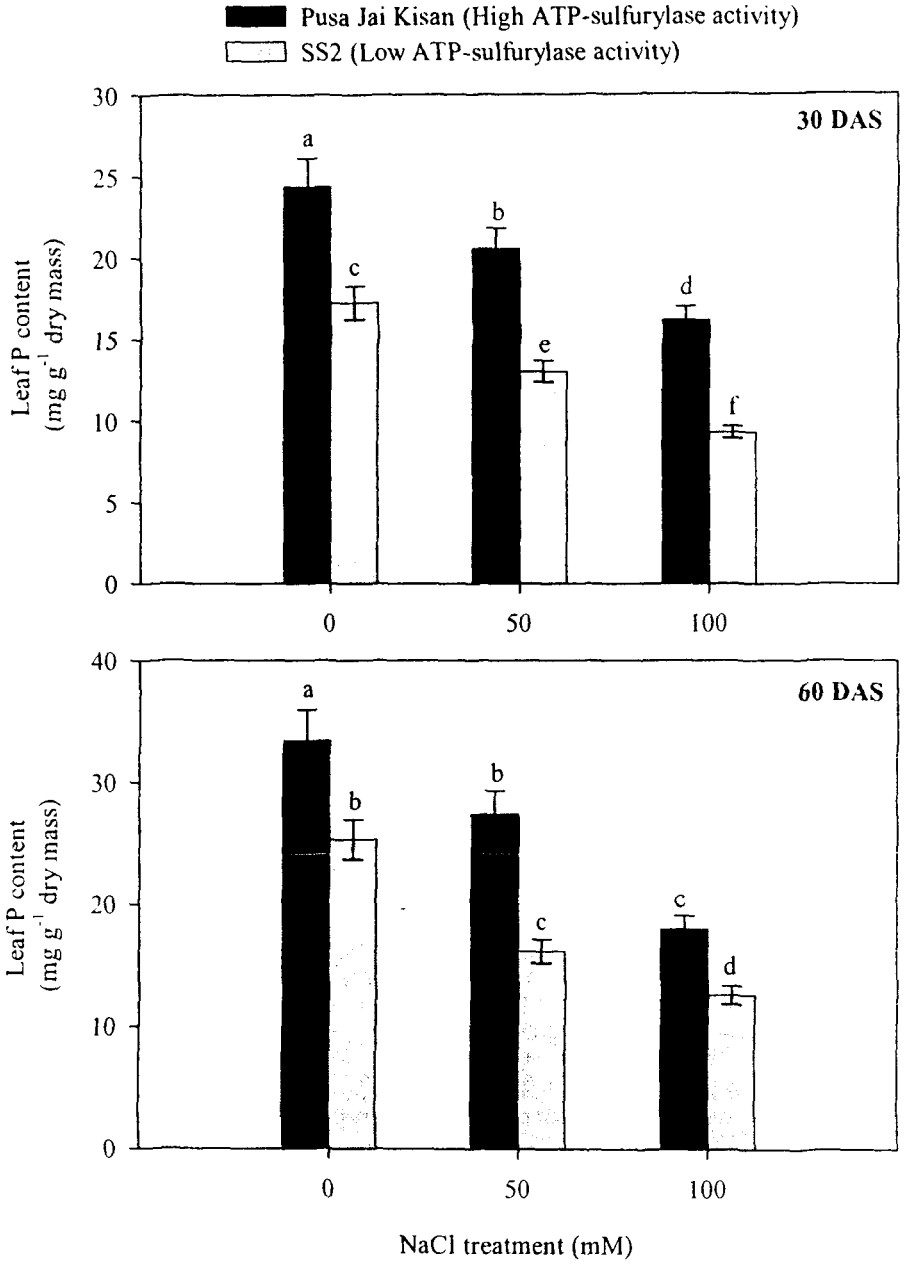


Figure 15. Effect of NaCl treatments on leaf P content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

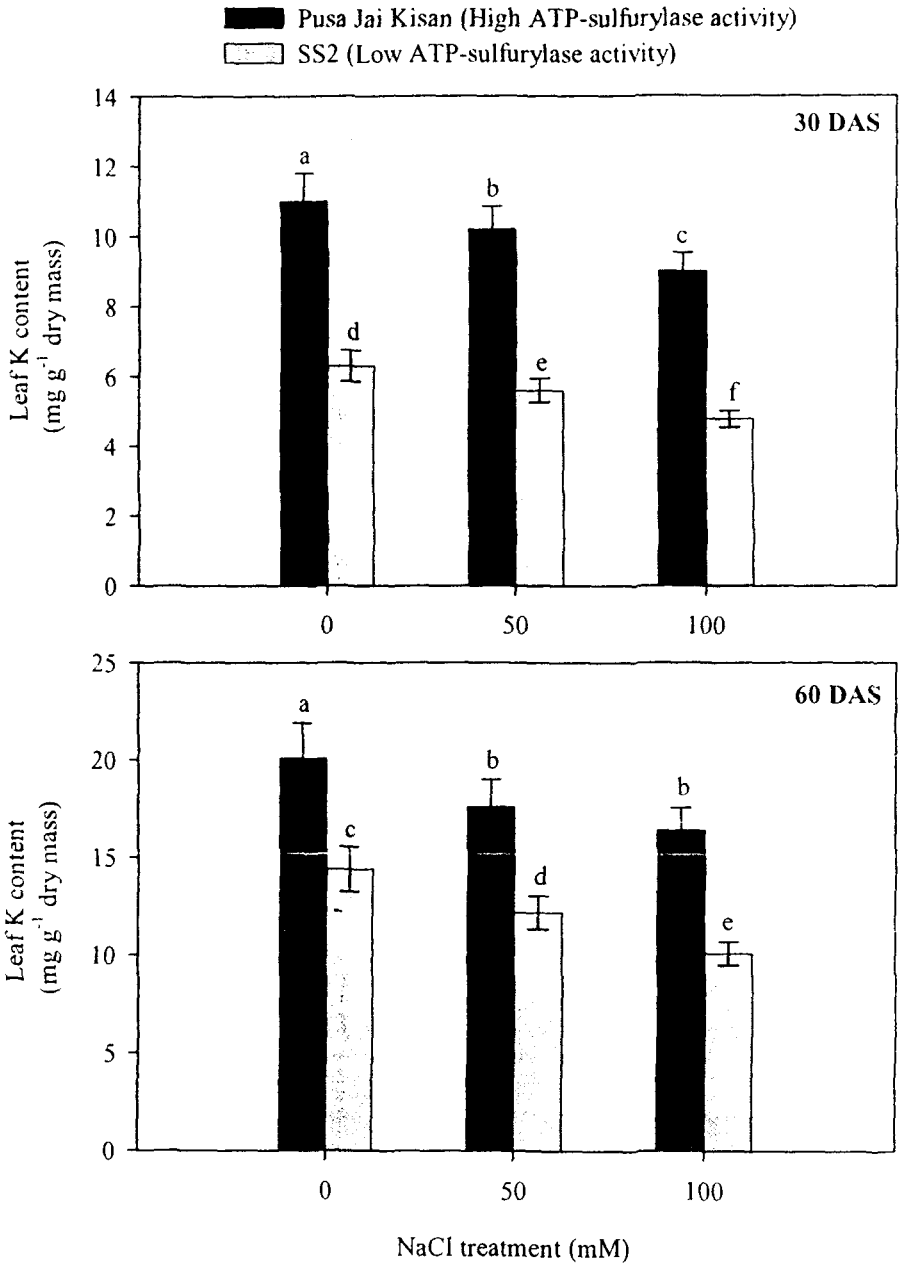


Figure 16. Effect of NaCl treatments on leaf K content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

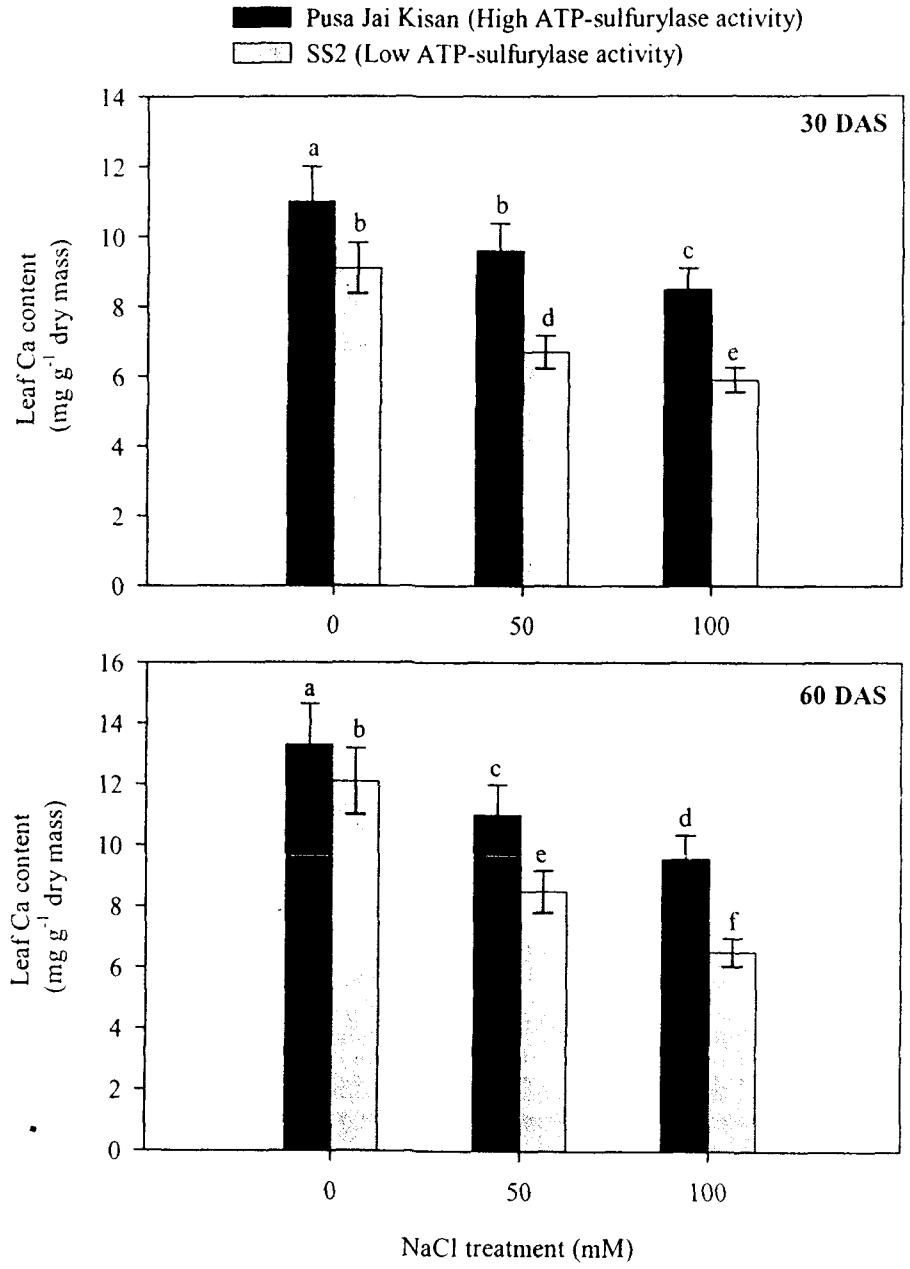


Figure 17. Effect of NaCl treatments on leaf Ca content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

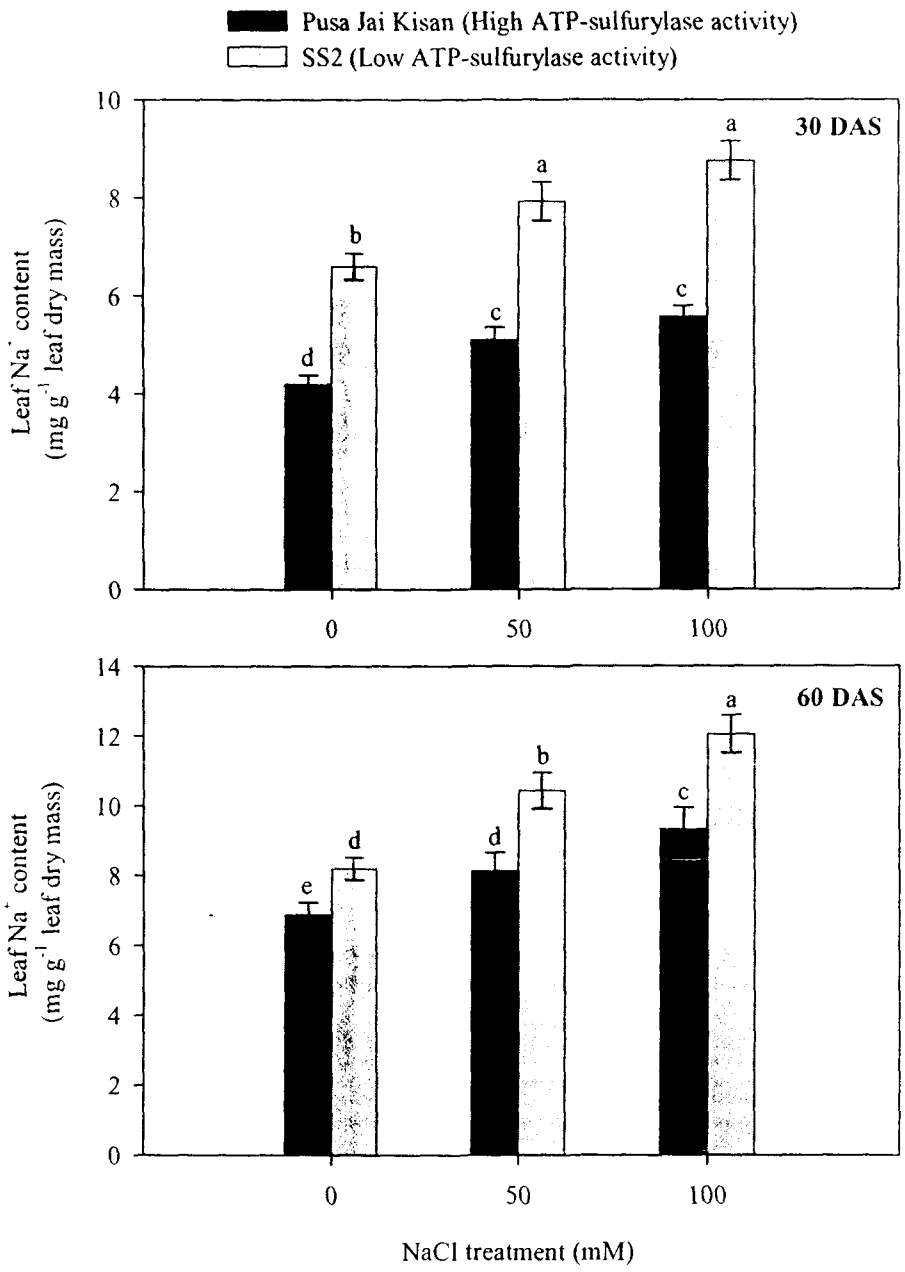


Figure 18. Effect of NaCl treatments on leaf Na^+ content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

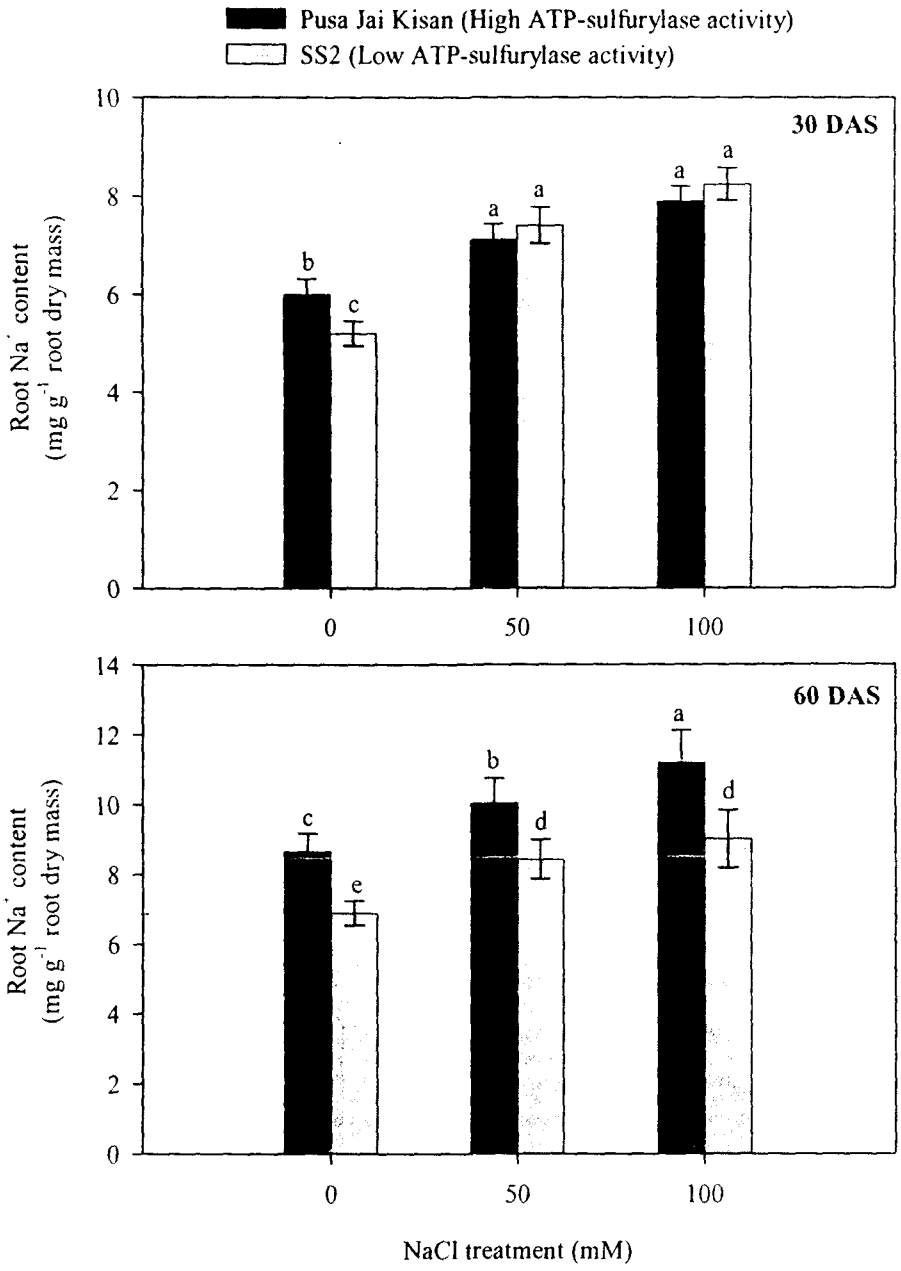


Figure 19. Effect of NaCl treatments on root Na^+ content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

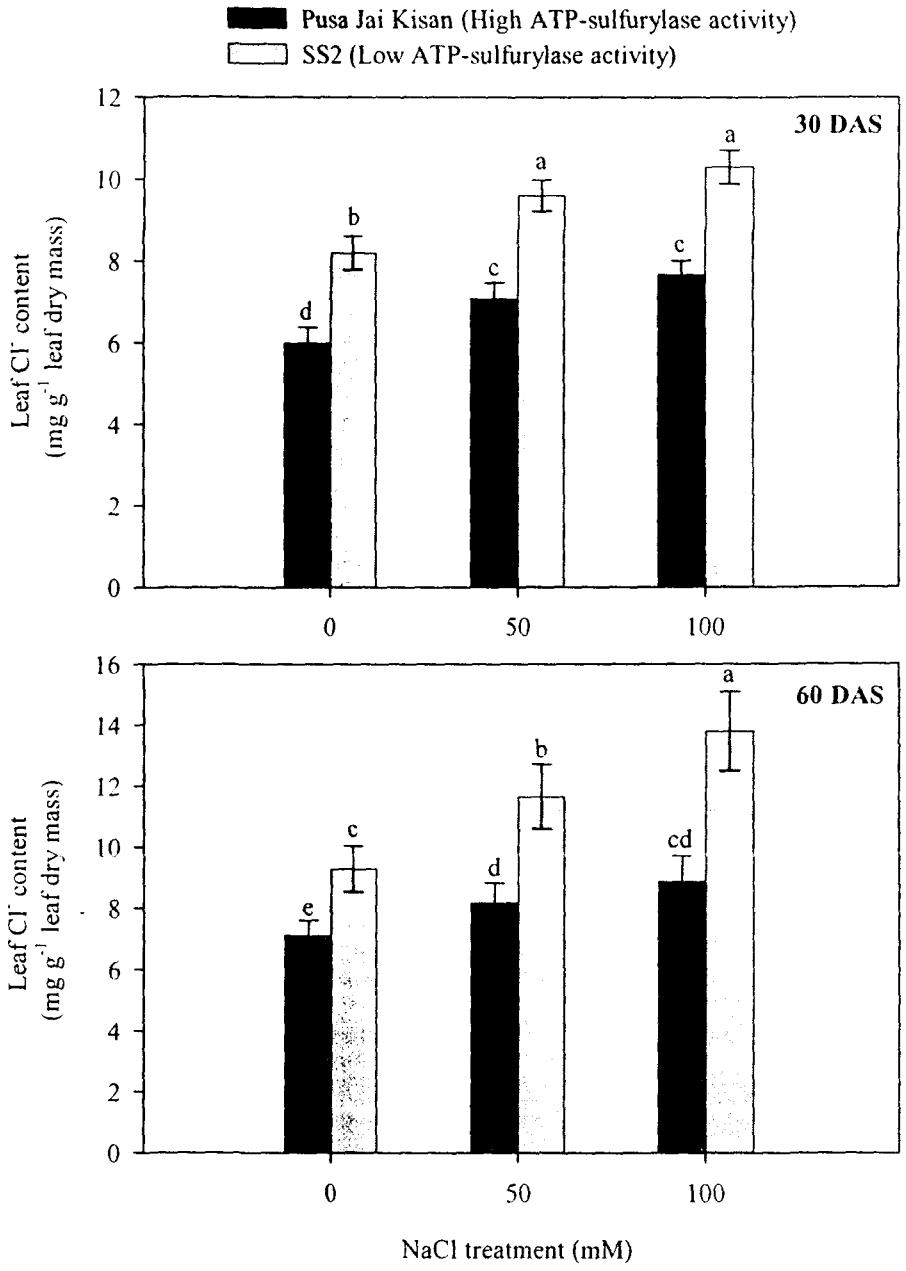


Figure 20. Effect of NaCl treatments on leaf Cl^- content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

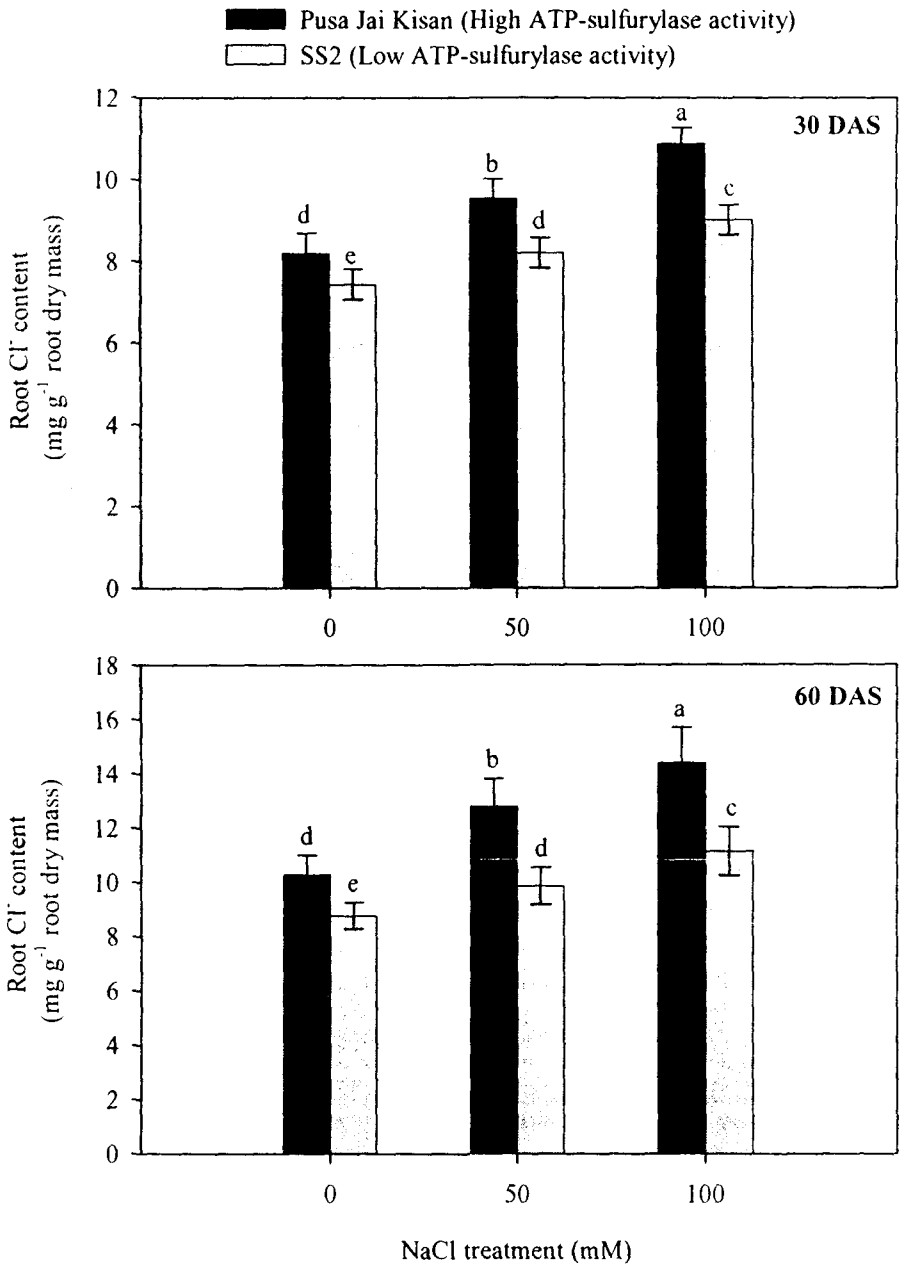


Figure 21. Effect of NaCl treatments on root Cl^- content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

control. In SS2, the above characteristics were increased by 21.2% and 25.6% at 30 DAS, 27.1% and 48.6% at 60 DAS in comparison with control.

4.2.5 Oxidative stress

Salinity stress increased the content of TBARS and H_2O_2, electrolyte leakage, membrane stability index and relative salt injury and the values were significantly higher than control in both the cultivars. The plants raised without NaCl showed equal content of TBARS and H_2O_2 and electrolyte leakage in both the cultivars. However, the values for these traits were significantly higher in SS2 than Pusa Jai Kisan under 50 and 100 mM NaCl (Figures 22-26).

In Pusa Jai Kisan, the content of TBARS and H_2O_2 and electrolyte leakage were increased by 256.1%, 178.3% and 37.63% at 30 DAS; 395.4%, 230.2% and 140.4%, respectively at 60 DAS due to 100 mM NaCl compared to the respective control. In SS2, the values for above characteristics were increased by 349.2%, 268.6% and 86.97% at 30 DAS; 591.7%, 300.9% and 131.5%, respectively at 60 DAS due to 100 mM NaCl compared to control.

Salinity stress significantly decreased the membrane stability index in both the cultivars, but the decrease was higher in SS2 than Pusa Jai Kisan. The decrease in membrane stability index with 100 mM NaCl in Pusa Jai Kisan was 13.7% and 18.6% at 30 and 60 DAS over their respective control. In SS2, this decrease was 41.2% and 44.1% at 30 and 60 DAS over their respective control.

Relative salt injury increased significantly with the increasing NaCl concentration and was greater in SS2 than Pusa Jai Kisan. A significant increase in relative salt injury in Pusa Jai Kisan due to 100 mM NaCl was 29.4% at 30 DAS and 55.6% at 60 DAS in comparison to control. In SS2, the increase in relative salt injury due to 100 mM NaCl was 71.6% and 105.6% at 30 and 60 DAS in comparison to control.

4.2.6 Enzymatic and Non-Enzymatic Antioxidants

4.2.6.1 Enzymatic antioxidants

Salinity stress enhanced the activity of antioxidative enzymes at both the sampling times (Figures 27-30). The activity of SOD increased with the increasing NaCl concentration and the extent of increase was greater in SS2 than Pusa Jai Kisan. In Pusa Jai Kisan, the activity of SOD was increased by 69.2% and 60.3% with 100 mM NaCl at 30 and 60 DAS over their respective control. The cultivar SS2 showed

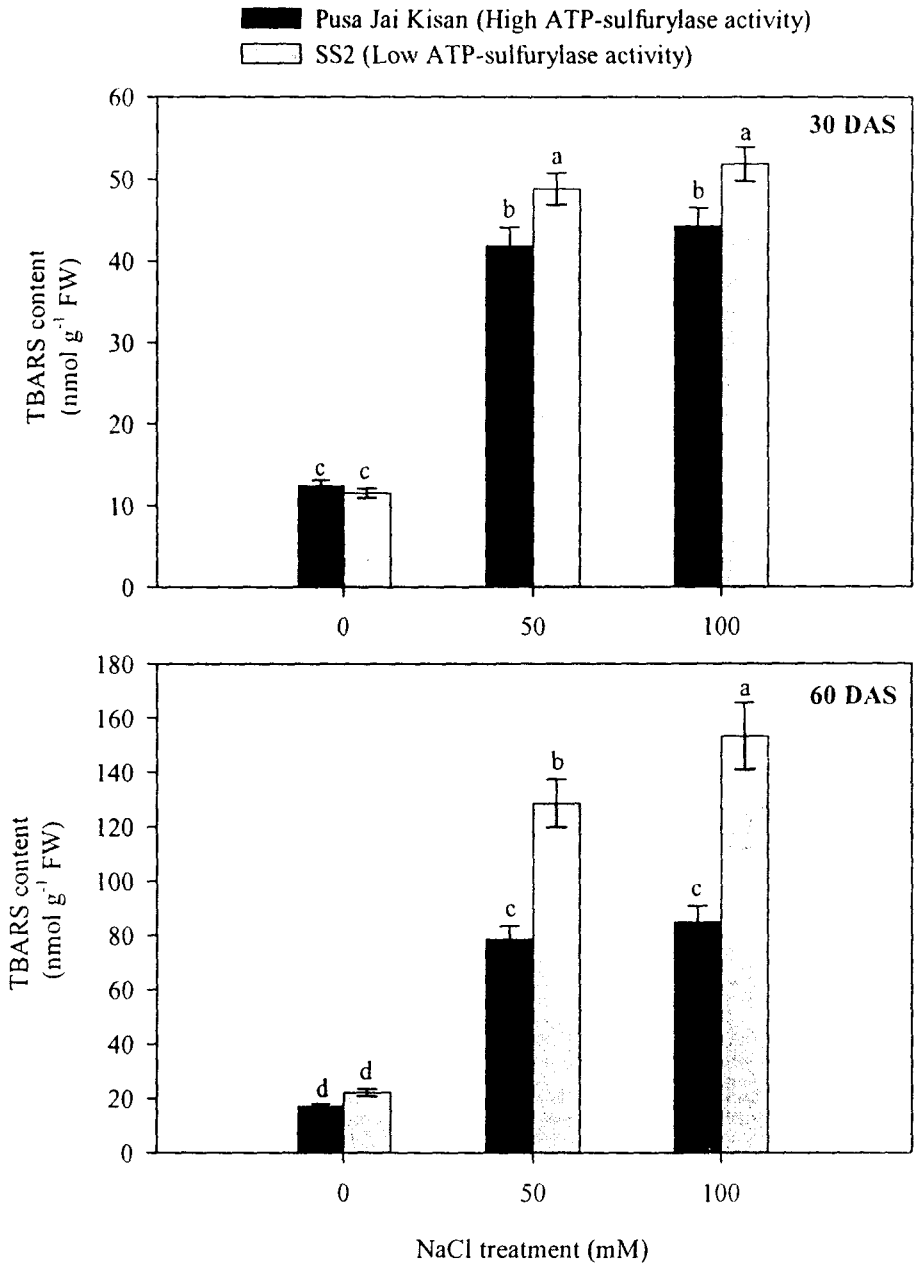


Figure 22. Effect of NaCl treatments on TBARS content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

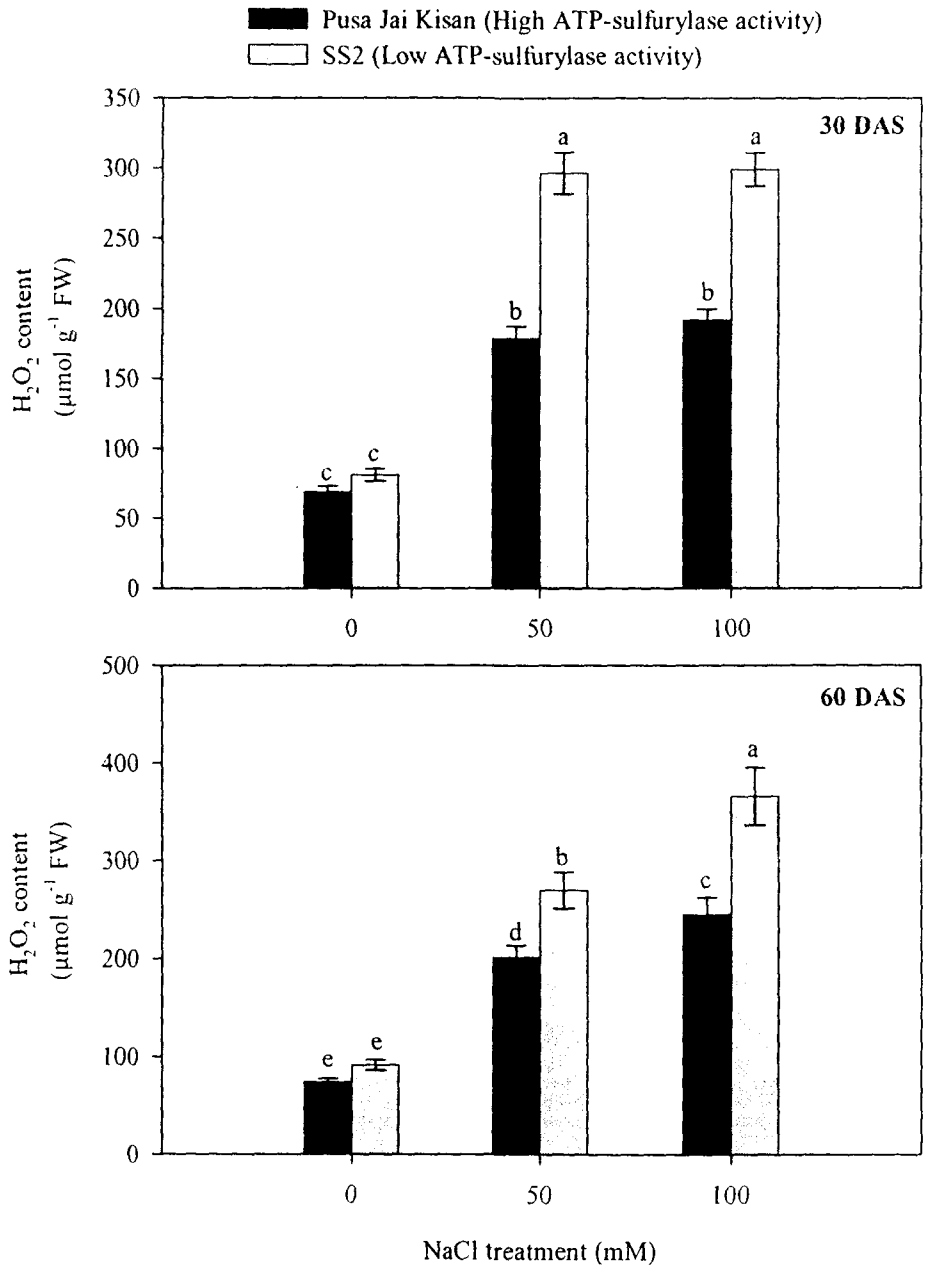


Figure 23. Effect of NaCl treatments on H_2O_2 content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

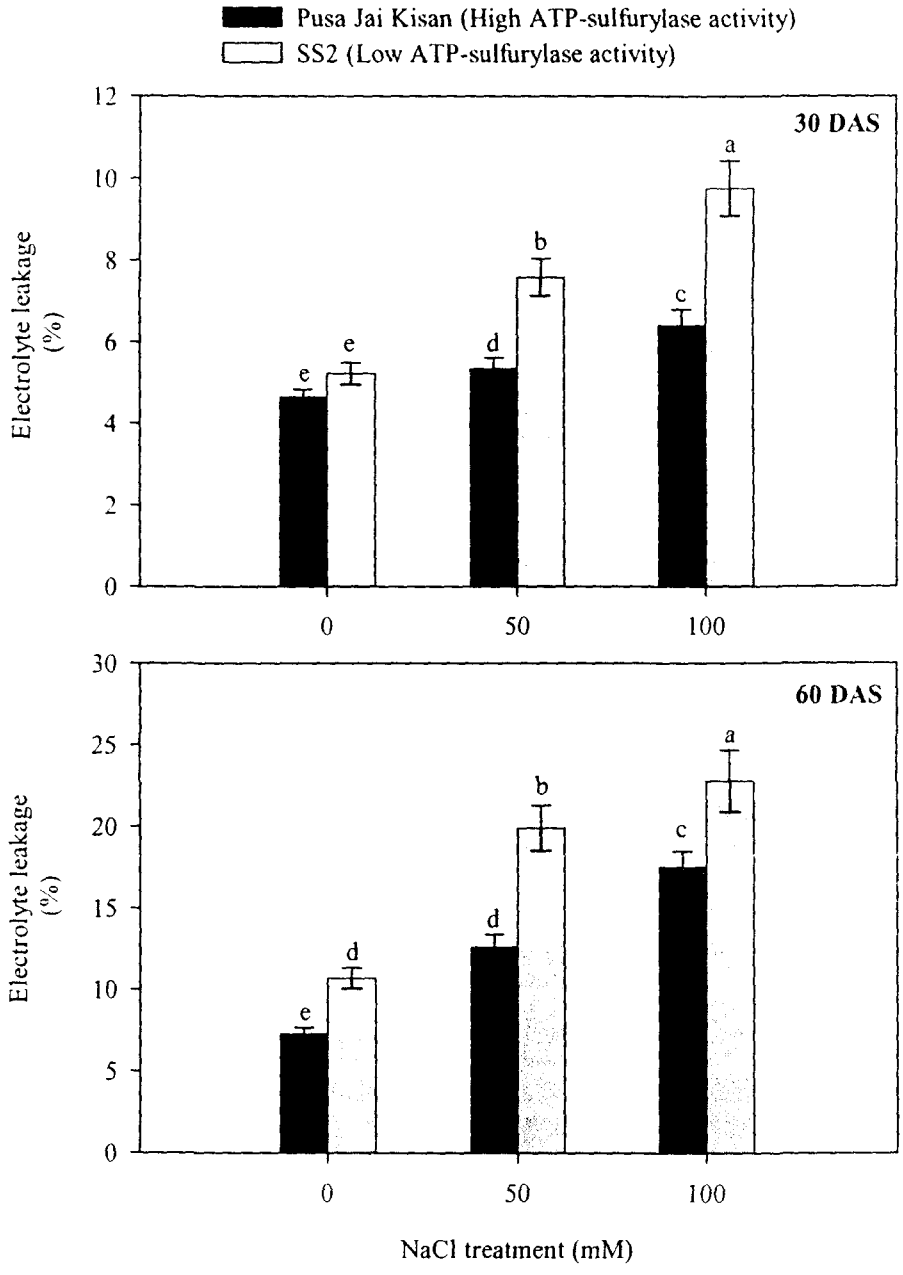


Figure 24. Effect of NaCl treatments on electrolyte leakage of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

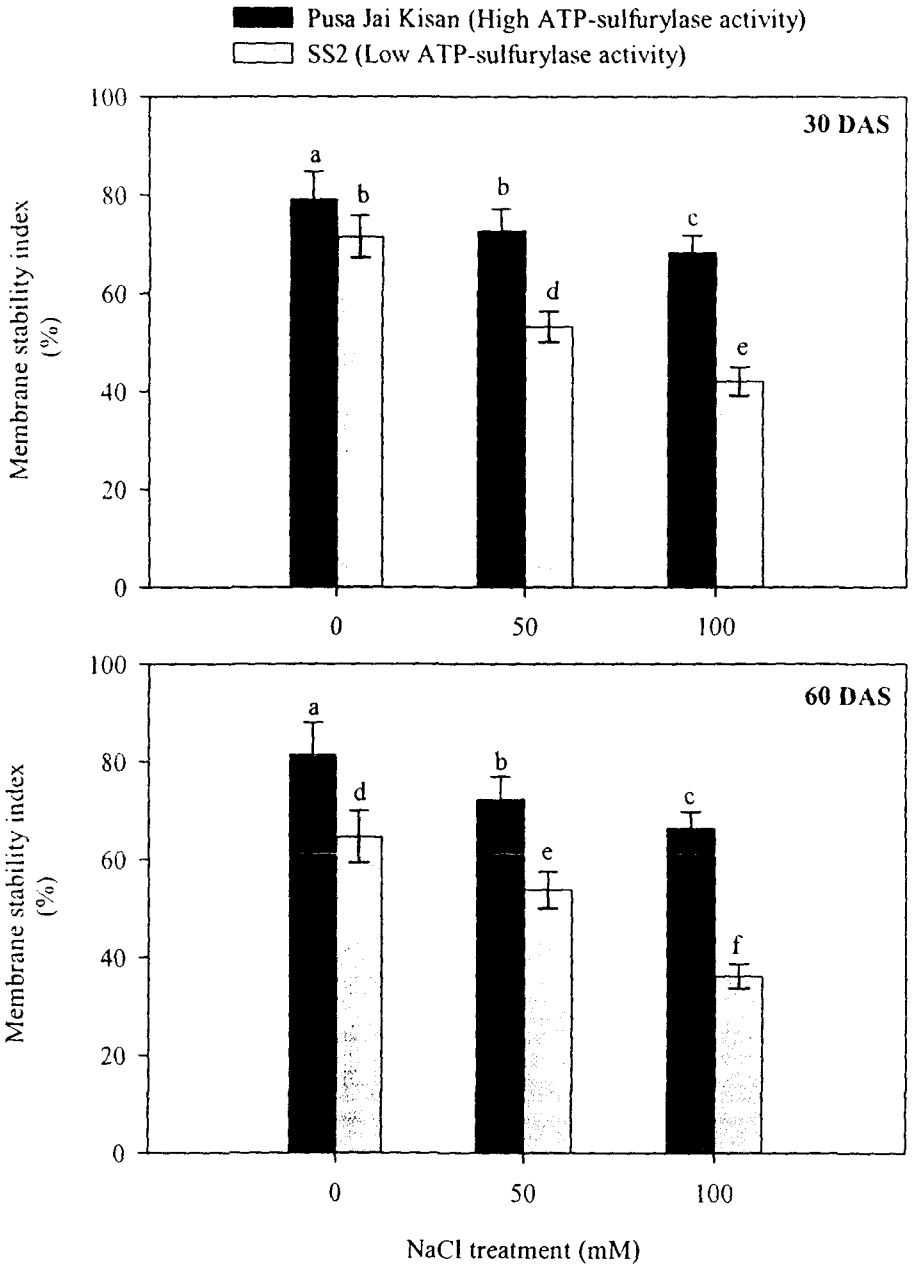


Figure 25. Effect of NaCl treatments on membrane stability index of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

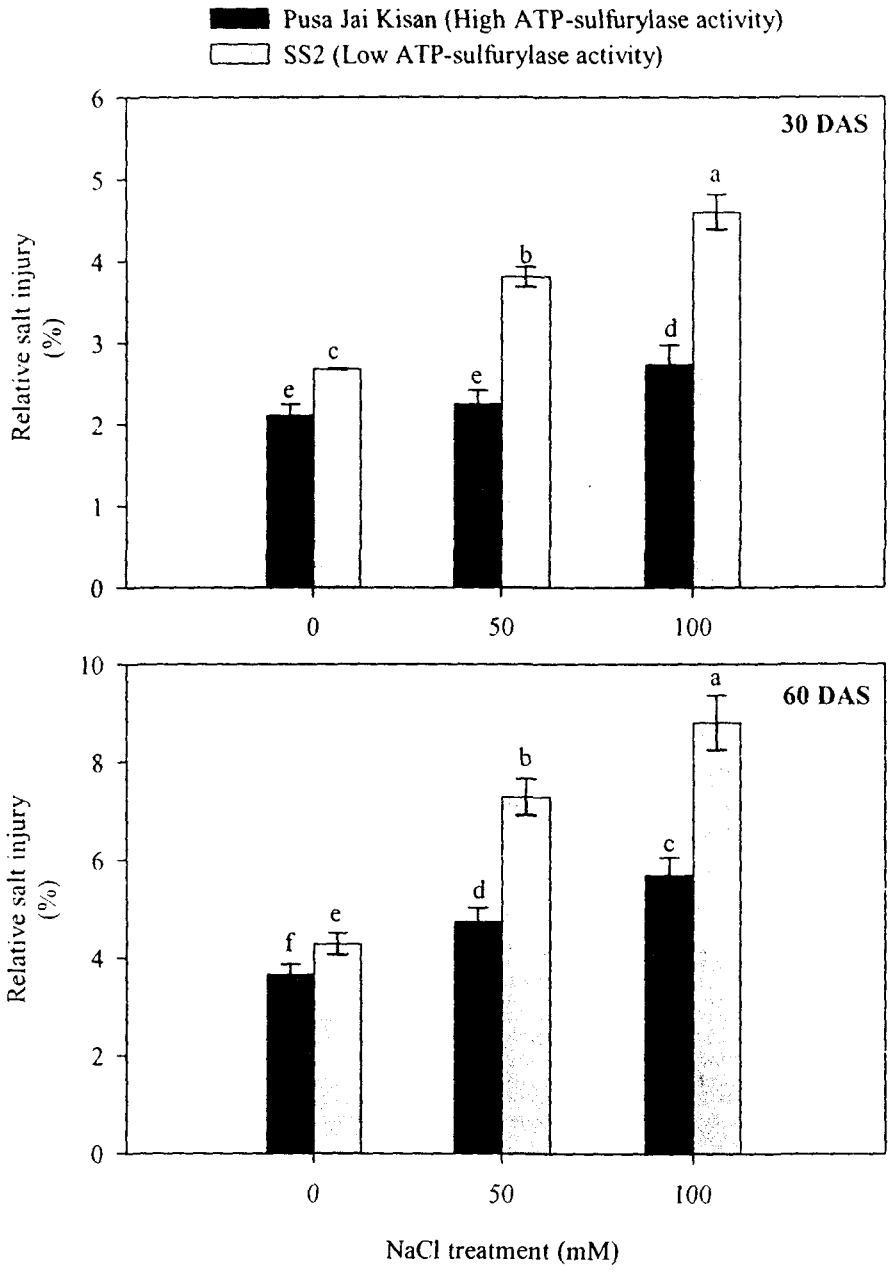


Figure 26. Effect of NaCl treatments on relative salt injury of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

higher increase of 104.6% and 76.8% with 100 mM NaCl at 30 and 60 DAS, respectively over their respective control.

The activity of CAT, APX and GR was higher in Pusa Jai Kisan than SS2. In Pusa Jai Kisan, the activity of these enzymes increased by 73.0%, 224.6% and 62.3% at 30 DAS and 125.0%, 237.8% and 161.4% at 60 DAS, respectively with 100 mM NaCl in comparison to control. In SS2, the activity of these enzymes was increased by 38.2%, 98.0% and 34.3% at 30 DAS, and 24.4%, 150.8% and 67.2% at 60 DAS, respectively with 100 mM NaCl in comparison to control (Figures 27-30).

4.2.6.2 Non-enzymatic antioxidants

Salinity stress decreased reduced ascorbate content significantly in both the cultivars. The decrease was greater in SS2 than Pusa Jai Kisan. However, the effect of 50 and 100 mM NaCl on reduced ascorbate content did not differ significantly in both the cultivars. Contrarily, reduced glutathione content increased significantly with the increase in NaCl level in both the cultivars. The increase was higher in Pusa Jai Kisan than SS2 at 100 mM NaCl (Figures 31-32).

Reduced ascorbate content was decreased by 37.7% and 45.8% with 100 mM NaCl at 30 and 60 DAS in Pusa Jai Kisan in comparison to control. In SS2, the content was decreased by 56.6% and 70.8% with 100 mM NaCl at 30 and 60 DAS in comparison to control.

The increase in reduced glutathione content in Pusa Jai Kisan due to 100 mM NaCl was 329.6% and 92.3% at 30 and 60 DAS over the control. In SS2, the content was increased by 204.4% and 68.3% at 30 and 60 DAS due to 100 mM NaCl in comparison to control.

4.2.7 Growth characteristics

Plant dry mass and leaf area decreased with the increasing NaCl concentration at both the sampling times. Maximum decrease in plant dry mass and leaf area was found with 100 mM NaCl in both the cultivars (Figures 33-35).

In Pusa Jai Kisan, the treatment of 100 mM NaCl decreased plant dry mass by 28.9% and leaf area by 26.3% at 30 DAS in comparison to control. At 60 DAS, the decreases in the above characteristics due to 100 mM NaCl were 41.2% and 34.9%, respectively in comparison to control. Low ATP-sulfurylase activity cultivar SS2 showed decrease in plant dry mass and leaf area of 51.0% and 36.8% with 100 mM NaCl at 30 DAS and 59.1% and 45.9% at 60 DAS in comparison to control.

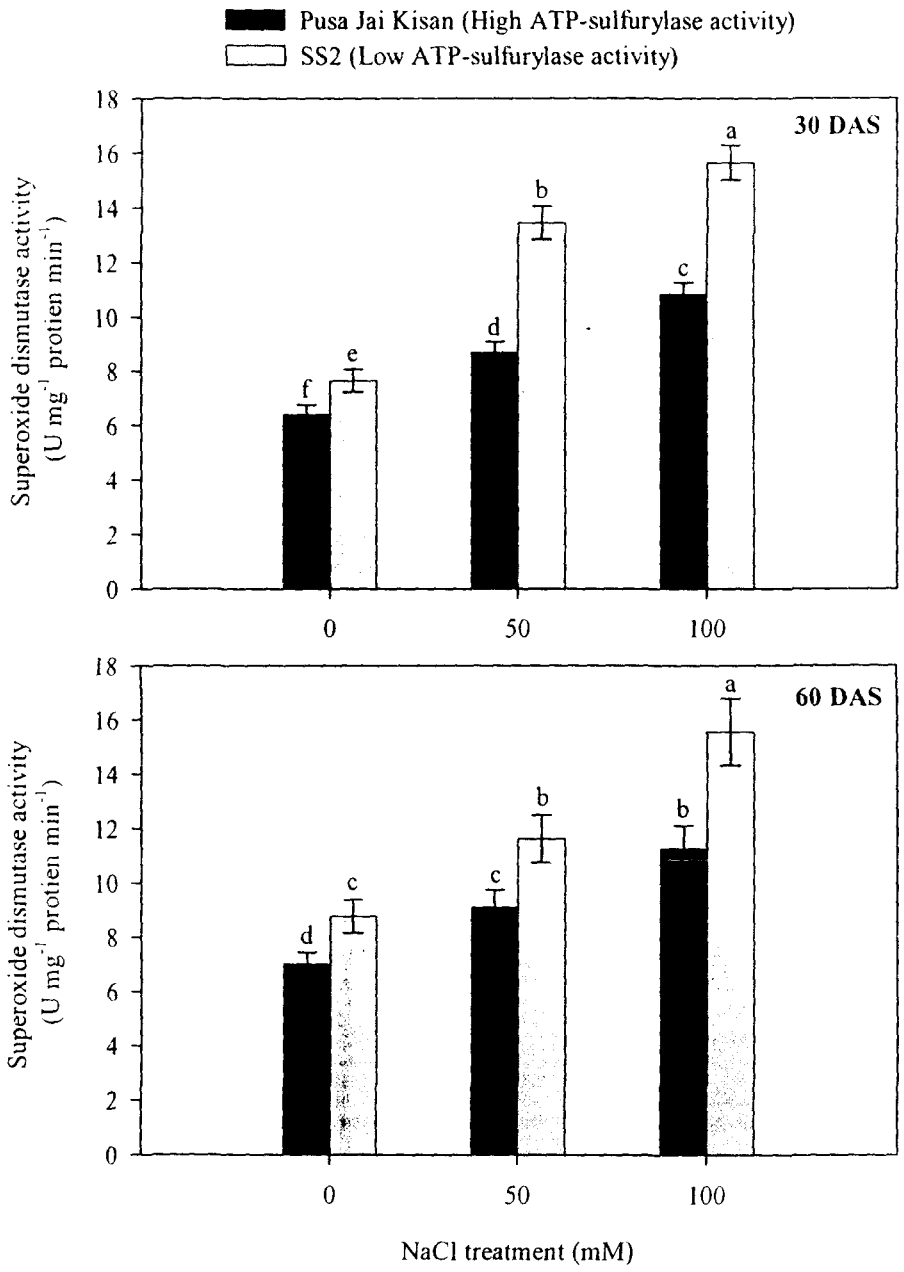


Figure 27. Effect of NaCl treatments on superoxide dismutase activity of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

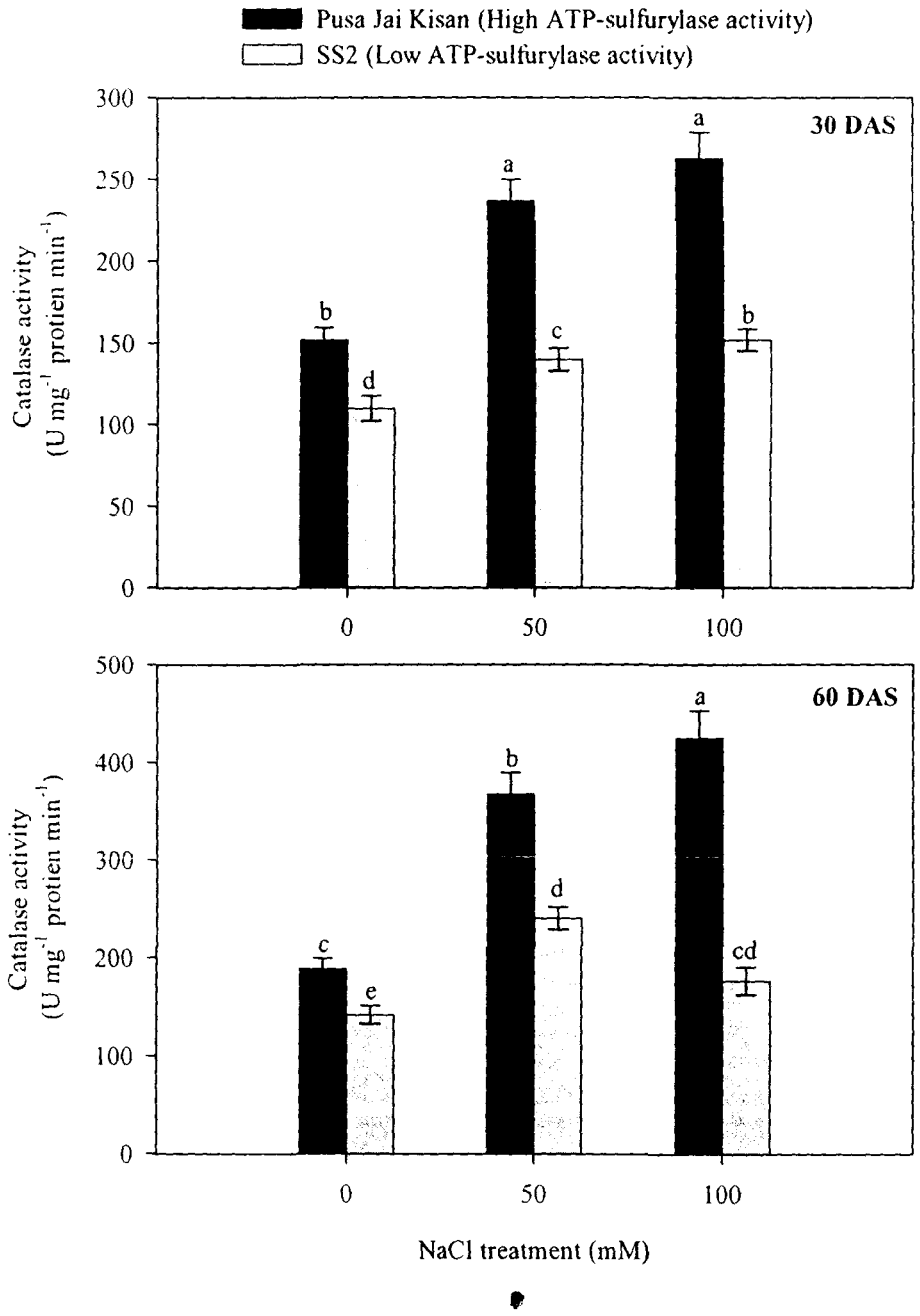


Figure 28. Effect of NaCl treatments on catalase activity of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

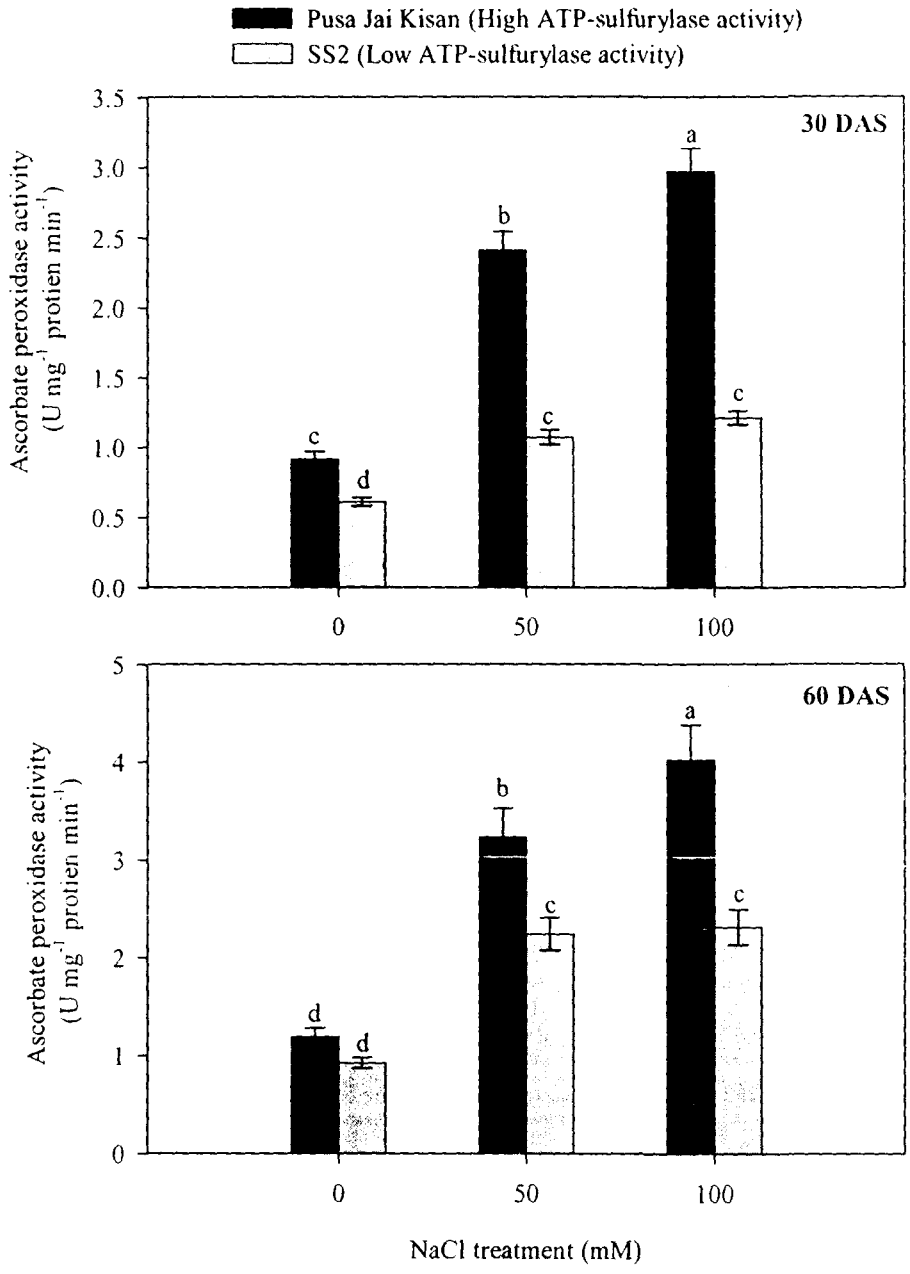


Figure 29. Effect of NaCl treatments on ascorbate peroxidise activity of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

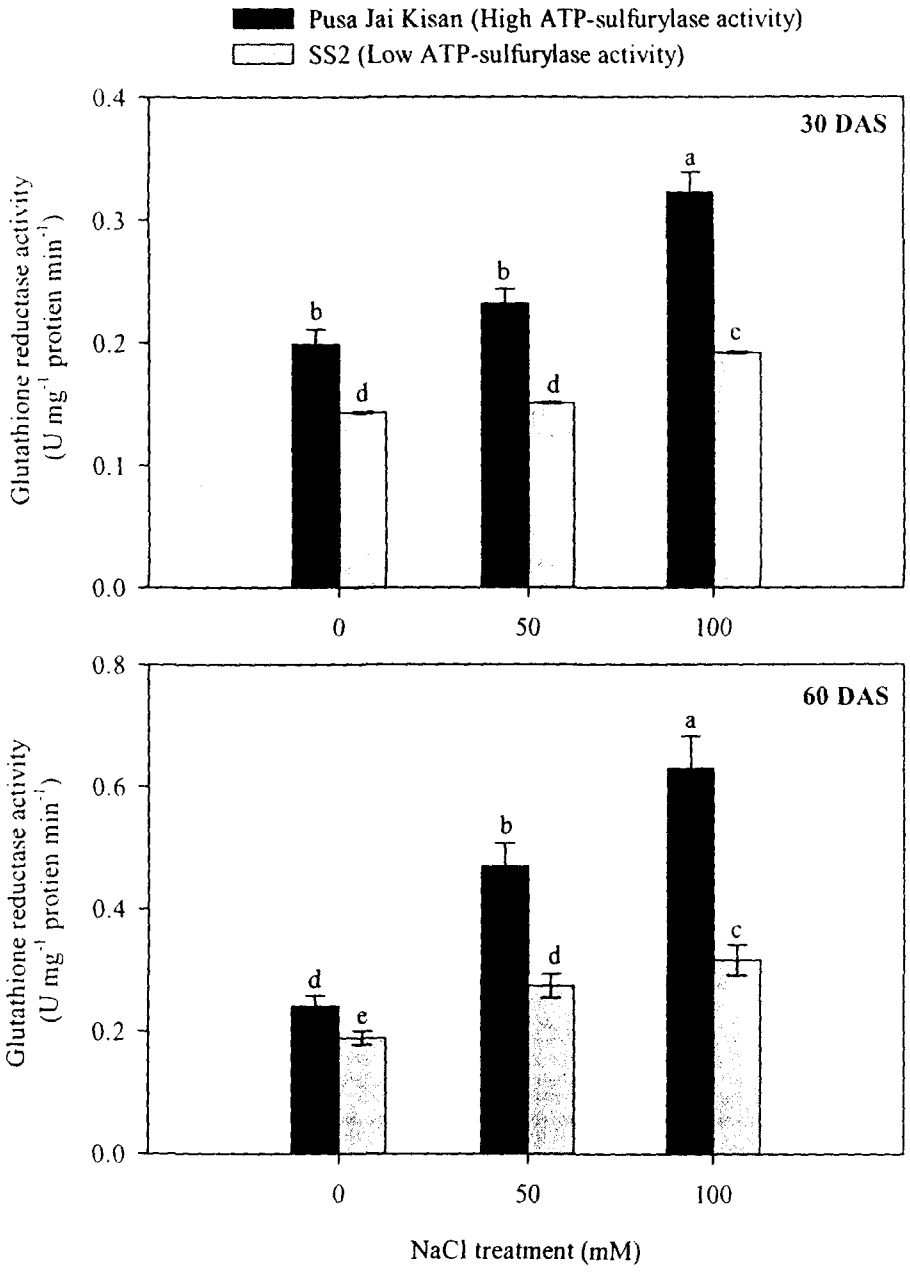


Figure 30. Effect of NaCl treatments on glutathione reductase activity of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

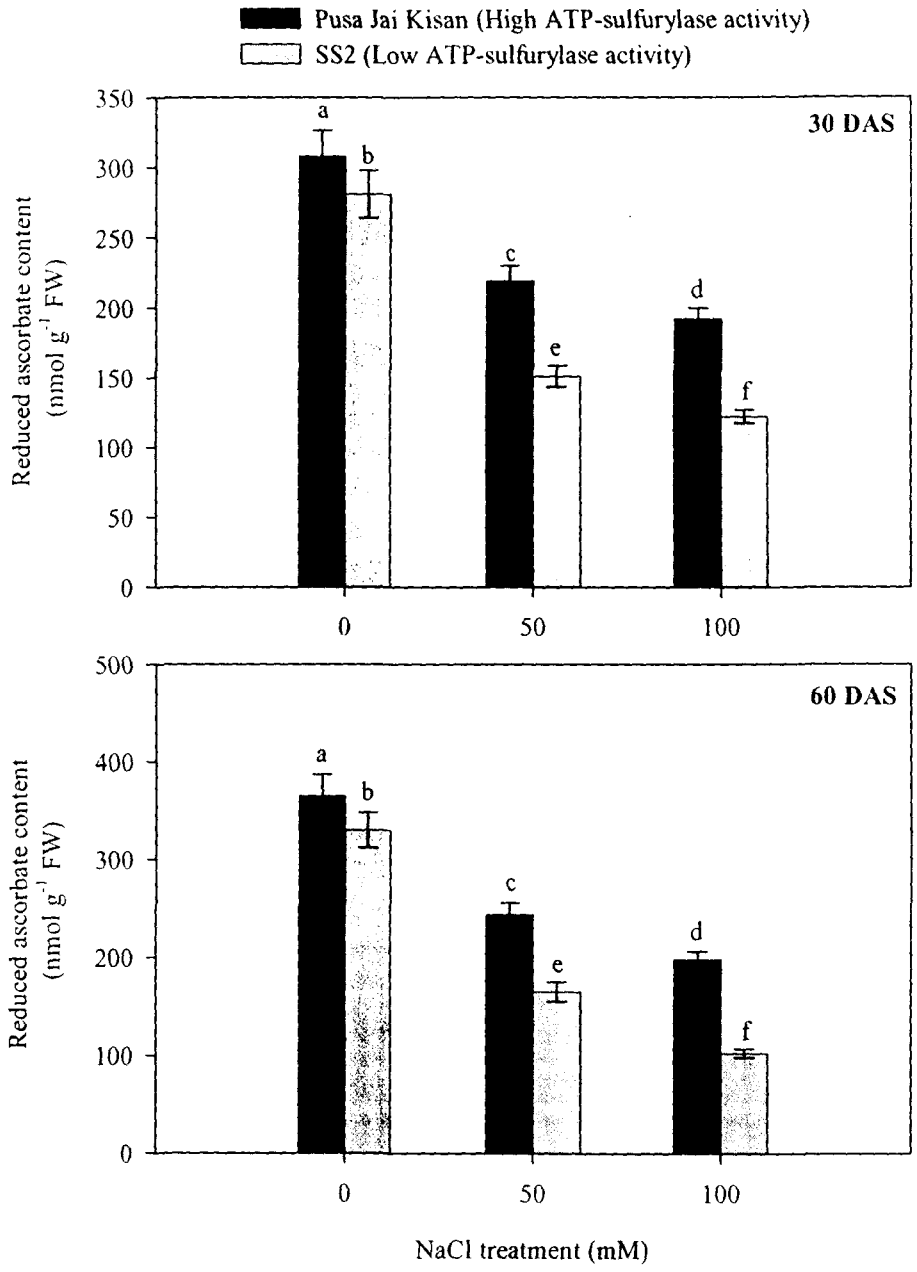


Figure 31. Effect of NaCl treatments on reduced ascorbate content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

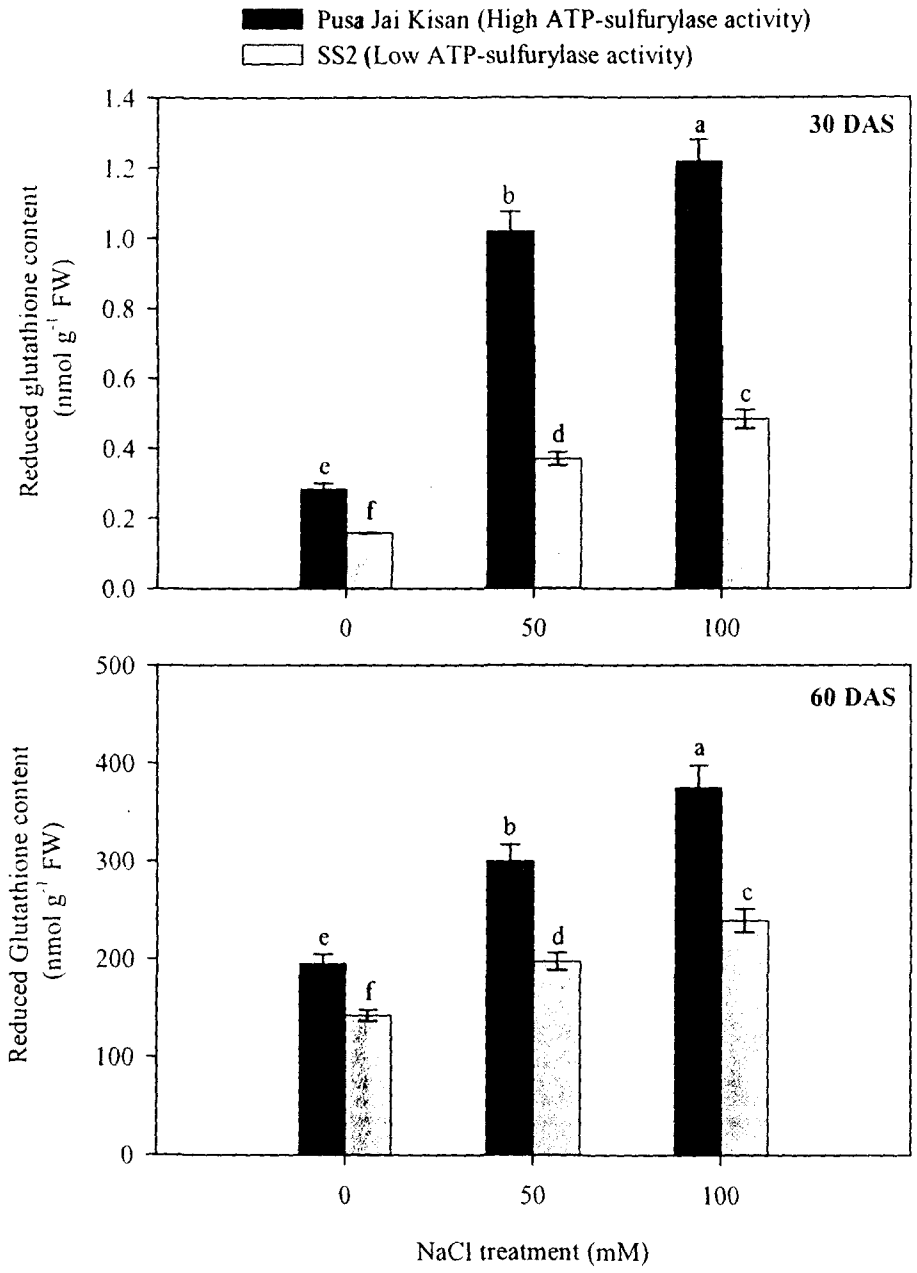


Figure 32. Effect of NaCl treatments on reduced glutathione content of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

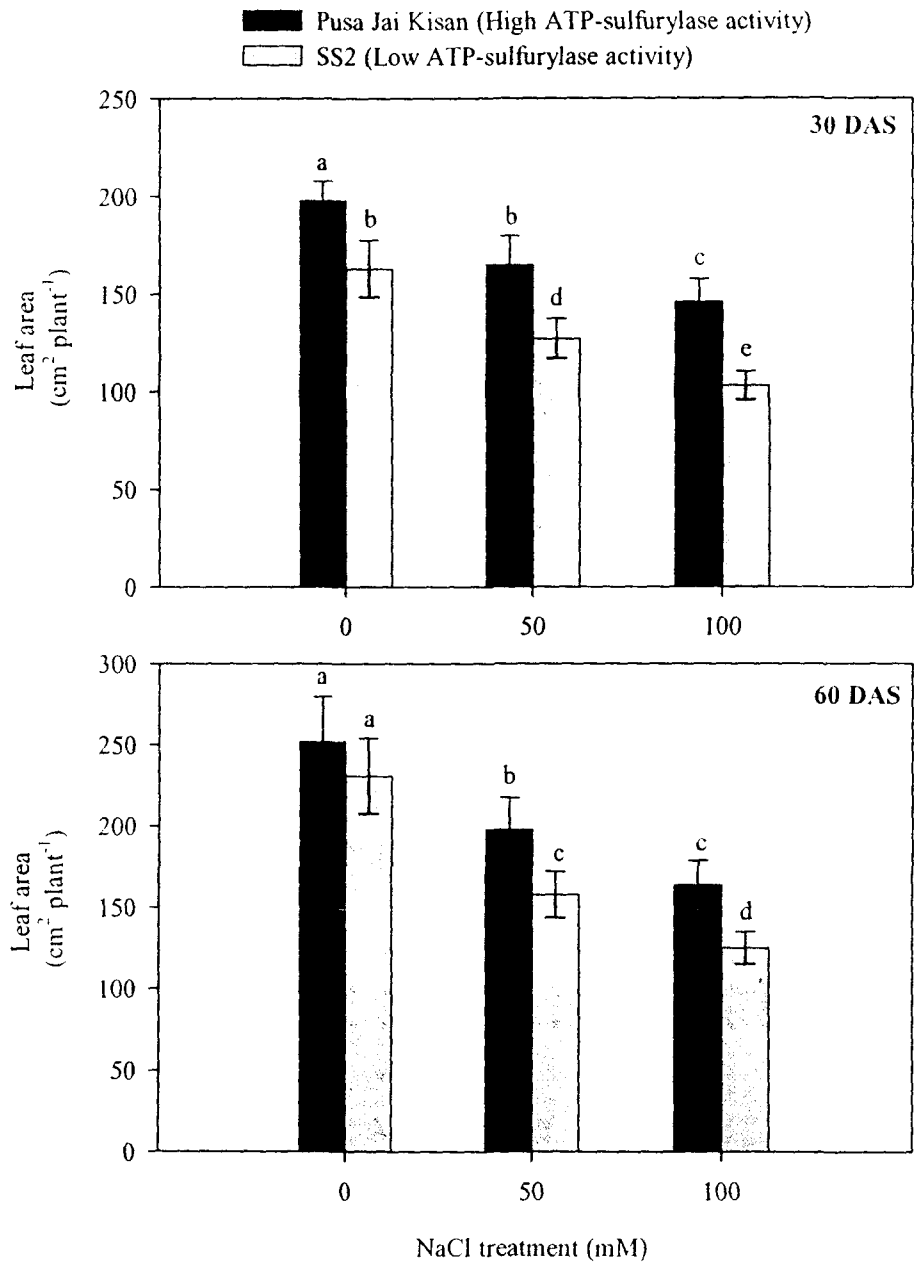


Figure 33. Effect of NaCl treatments on leaf area of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

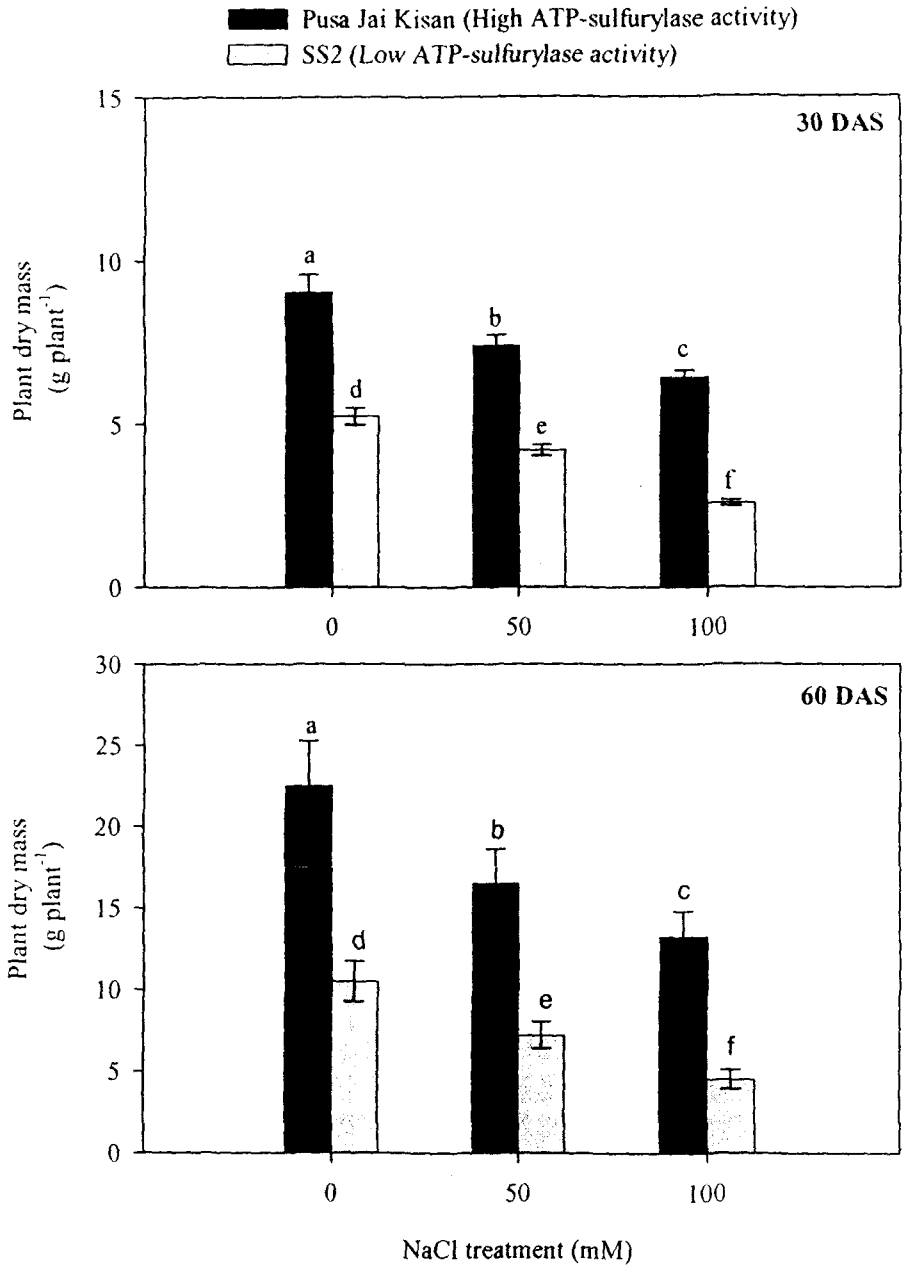


Figure 34. Effect of NaCl treatments on plant dry mass of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

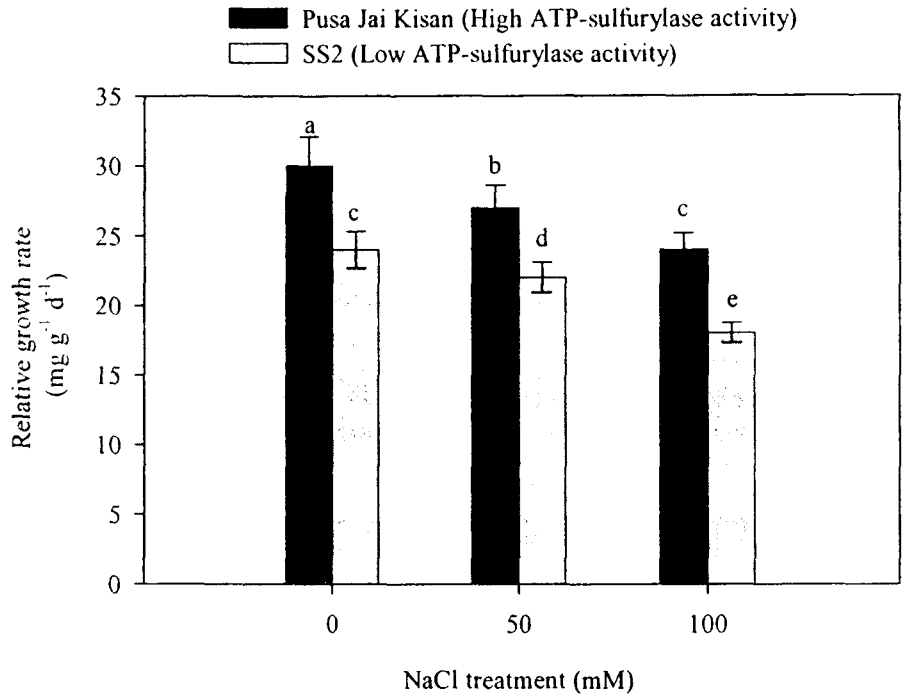


Figure 35. Effect of NaCl treatments on relative growth rate of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

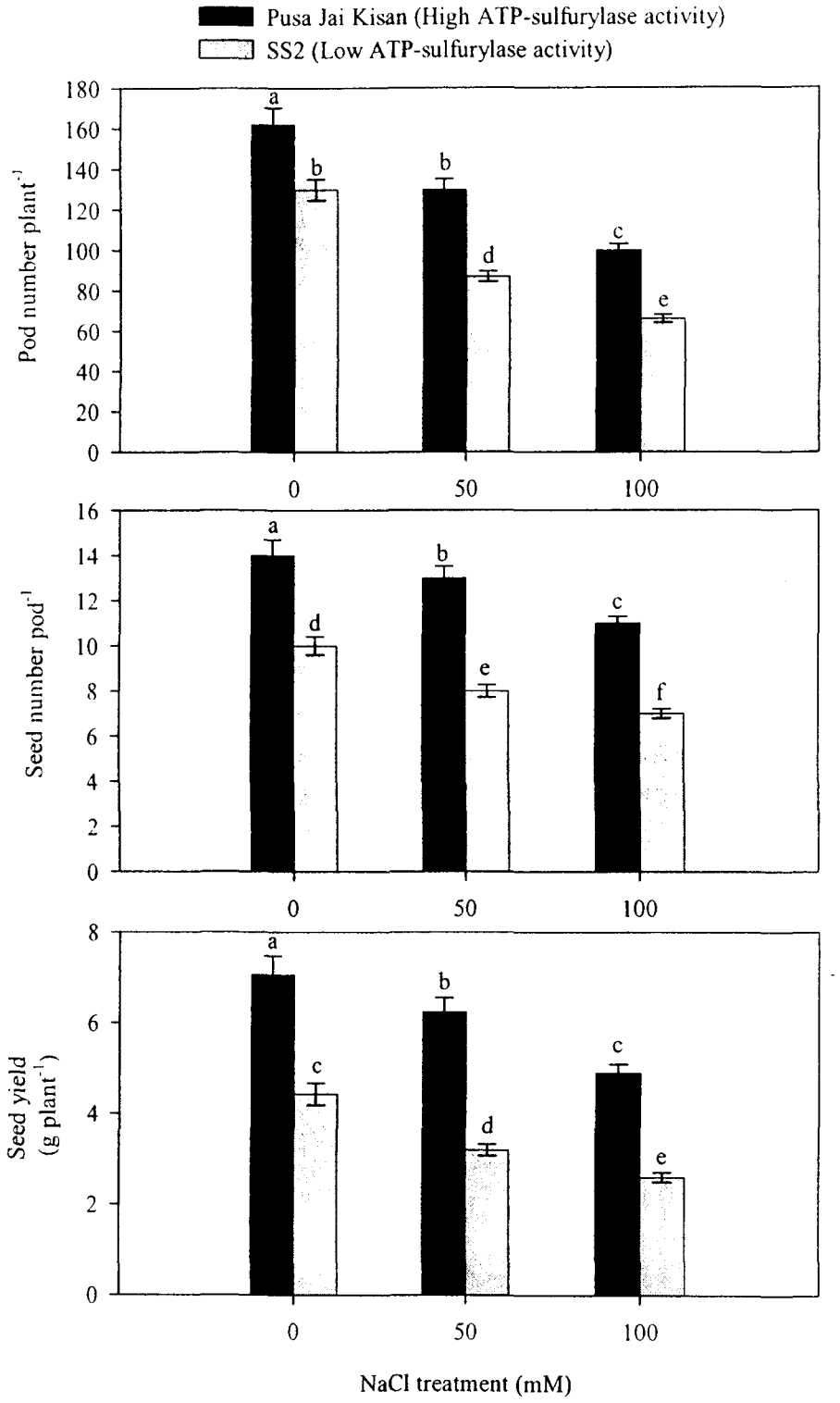


Figure 36. Effect of NaCl treatments on pod number per plant, seed number per plant and seed yield of two cultivars of mustard (*Brassica juncea* L.) at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

In Pusa Jai Kisan and SS2, relative growth rate was decreased by 20.0% and 25.0%, respectively with 100 mM NaCl in comparison to control.

4.2.8 Yield characteristics

Salinity stress significantly decreased the yield characteristics (pod number per plant, seed number per plant and seed yield per plant) at harvest and the extent of decrease was greater in SS2 than Pusa Jai Kisan with 100 mM NaCl treatment.

Pod number, seed number and seed yield per plant of Pusa Jai Kisan were decreased by 38.3%, 21.4% and 30.5%, respectively due to 100 mM NaCl over the control. In SS2, the decrease in these characteristic was 49.2%, 30.0% and 41.3%, respectively over the control (Figure 36).

4.2.9 Summary of Experiment 2

- The effect of 100 mM NaCl decreased the photosynthetic, growth and yield characteristics maximally. The effect of 100 mM NaCl was more conspicuous in SS2 compared to Pusa Jai Kisan.
- The cultivar SS2 (low ATP-sulfurylase activity) accumulated higher content of Na^+ and Cl^- in leaf than root. SS2 also showed greater content of TBARS and H_2O_2 and higher decrease in growth, photosynthetic traits and leaf water potential and osmotic potential than Pusa Jai Kisan with salinity stress. There exists a direct relationship of the content of leaf and root Na^+ and Cl^- with NaCl-accrued increase in oxidative stress (in terms of increase in level of TBARS and H_2O_2), modulation of plant antioxidant defense system and hence also with the reductions in growth, photosynthesis and yield and its attributes in both the cultivars.
- Pusa Jai Kisan exhibited lower induction of SOD but higher induction of CAT, APX, GR and GSH resulting in rapid detoxification of H_2O_2 produced under salinity stress in comparison to SS2.
- Pusa Jai Kisan cultivar with high ATP-sulfurylase activity showed greater tolerance to salinity stress as the result of its capacity to accumulate Na^+ and Cl^- in root, higher water potential, efficient antioxidant system and higher glutathione content. These characteristics of Pusa Jai Kisan helped in protecting the photosynthetic capacity and maintaining higher plant dry mass.

4.3 Experiment 3: Effect of Sulfur in the Amelioration of Salinity stress with Mustard Types Differing in ATP-Sulfurylase Activity

Experiment 3 was conducted on the basis of findings of Experiment 2. As observed in Experiment 2, maximum reductions in the observed characteristics were found with 100 mM NaCl. The present experiment was aimed at studying the effect of 1 and 2 mM SO_4^{2-} in the alleviation of 100 mM NaCl-induced reductions in Pusa Jai Kisan and SS2 cultivars of mustard at 30 and 60 DAS and yield characteristics at 120 DAS. Following sections present the results in detail (Figures 37-69).

4.3.1 ATP-sulfurylase activity and S content

NaCl treatment significantly increased the ATP-sulfurylase activity and S content over control in both the cultivars at both the growth stages. Application of sulfur either alone or in combination with 100 mM NaCl further increased the ATP-sulfurylase activity and S content in both the cultivars, which were found greatest in Pusa Jai Kisan (Figures 37-38).

In Pusa Jai Kisan, the increase in ATP-sulfurylase activity and S content due to 100 mM NaCl was 38.6% and 31.1% at 30 DAS; 44.5% and 39.1% at 60 DAS, in comparison to the respective control. The increase in these traits in SS2 was 19.8% and 15.6% at 30 DAS; 22.8% and 18.8% at 60 DAS in comparison to the respective control.

Application of sulfur alone as 1 mM SO_4^{2-} to Pusa Jai Kisan exhibited increased ATP-sulfurylase activity and S content by 63.4% and 63.6% at 30 DAS and 76.5% and 70.1% at 60 DAS over control. Higher S application (2 mM SO_4^{2-}) to Pusa Jai Kisan proved less effective and showed comparatively lesser increase of 30.7% and 35.0% in ATP-sulfurylase activity and S content at 30 DAS, 36.0% and 46.5% at 60 DAS in comparison to control. In SS2, the ATP-sulfurylase activity and S content increased by 35.4% and 46.4% at 30 DAS and 46.2% and 58.4% at 60 DAS due to 1 mM SO_4^{2-}, while 4.2% and 18.9% increase at 30 DAS and 11.1% and 26.5% increase at 60 DAS was noted compared to control.

Application of sulfur in the presence of NaCl showed higher ATP-sulfuylase activity and S content in comparison to the treatment of 100 mM NaCl or 2 mM SO_4^{2-} alone. However, 2 mM SO_4^{2-} in presence of NaCl showed higher ATP-sulfurylase activity and S content than 1 mM SO_4^{2-}. In Pusa Jai Kisan, ATP-sulfurylase activity and S content were increased by 53.6% and 57.5% at 30 DAS, 69.1% and 67.0% at 60

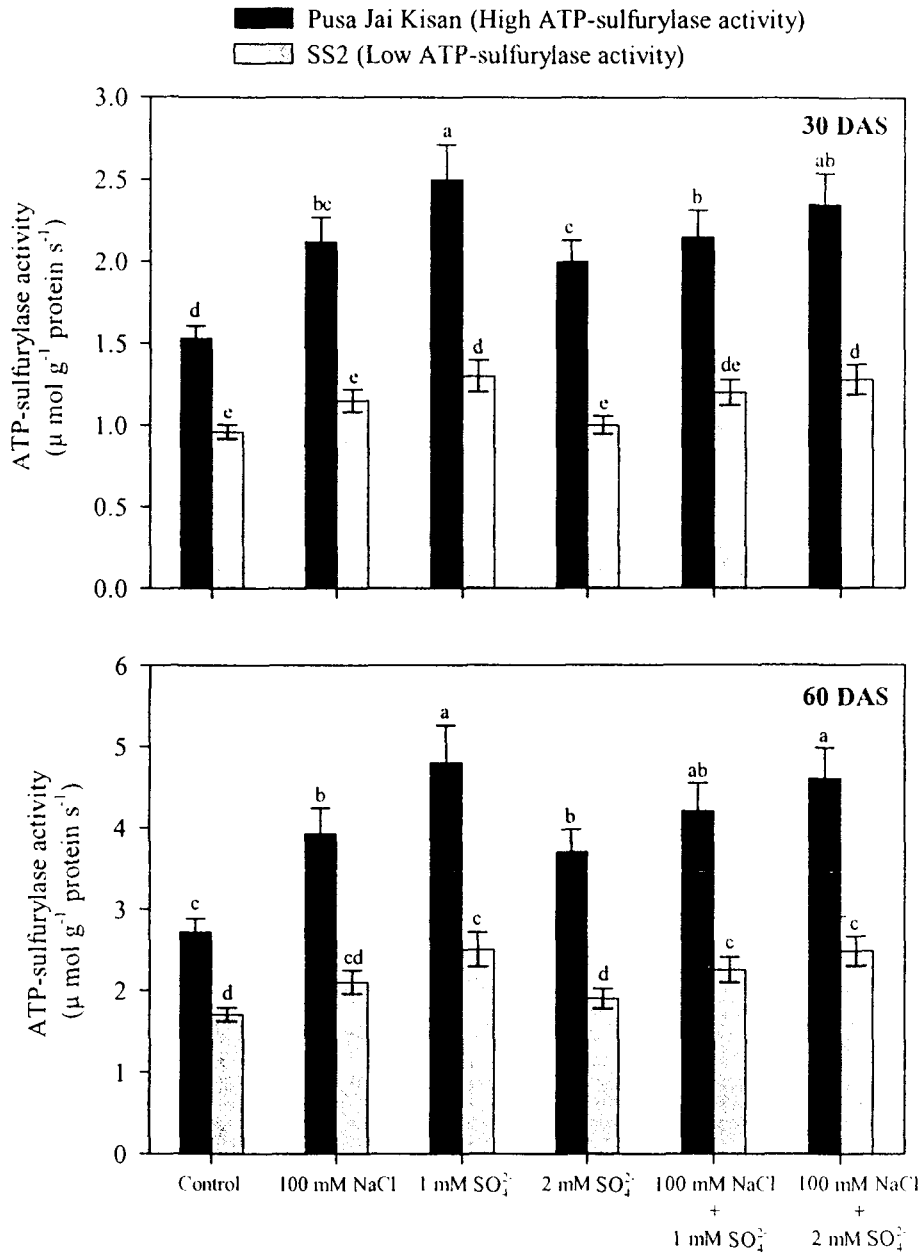


Figure 37. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on ATP-sulfurylase activity at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

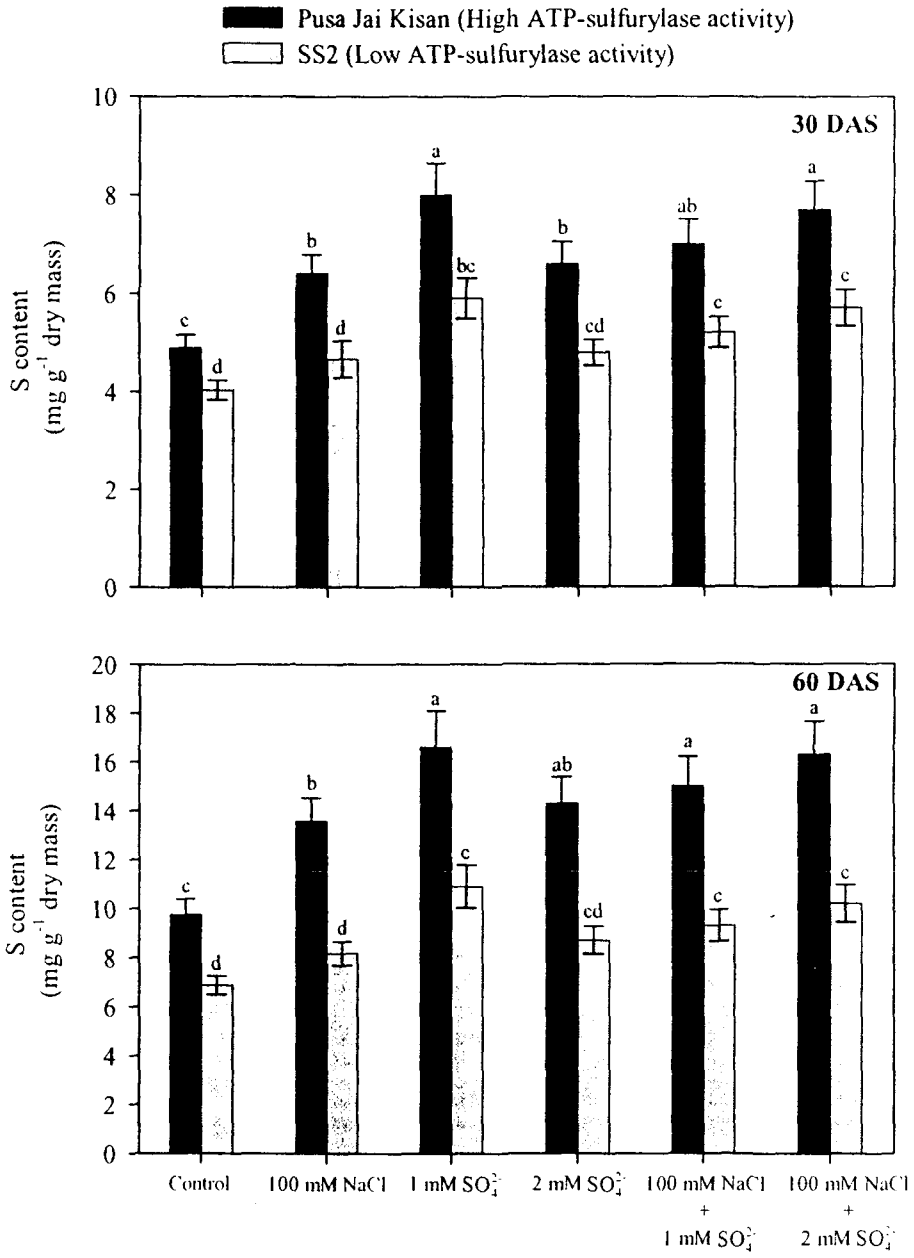


Figure 38. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on S content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

DAS. In SS2, these traits were increased by 33.3% and 41.4% at 30 DAS and 45.0% and 54.1% at 60 DAS due to 2 mM SO_4^{2-} in presence of NaCl in comparison to control. Application of 1 mM SO_4^{2-} in presence of NaCl increased ATP-sulfurylase activity and S content which were lesser than the increase induced by 2 mM SO_4^{2-} plus NaCl.

4.3.2 Photosynthetic characteristics

Photosynthetic characteristics (net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration and water-use efficiency) decreased significantly with 100 mM NaCl in both the cultivars at 30 and 60 DAS. However, the extent of decrease was higher in SS2 than Pusa Jai Kisan. The application of 2 mM SO_4^{2-} completely overcome the reductions caused by 100 mM NaCl on photosynthetic characteristics in both the cultivars, whereas 1 mM SO_4^{2-} application alleviated the NaCl-induced reductions to some extent in both the cultivars (Figures 39-44).

In Pusa Jai Kisan, the application of 100 mM NaCl reduced the net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration by 39.3%, 40.5% and 21.0% at 30 DAS, 45.7%, 45.8% and 26.8% at 60 DAS in comparison to control; while in SS2 greater reduction of 47.2%, 49.3% and 27.6% occurred at 30 DAS and 57.5%, 52.2% and 33.7% at 60 DAS over their respective control.

In both the cultivars application of 1 mM SO_4^{2-} alone increased the photosynthetic characteristics while 2 mM SO_4^{2-} exhibited reduction in photosynthetic characteristics in comparison to control. In Pusa Jai Kisan, 1 mM SO_4^{2-} increased the net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration by 28.5%, 14.8% and 7.7% at 30 DAS, 30.3%, 20.7% and 12.4% at 60 DAS; while in SS2 these were increased by 14.6%, 10.4% and 3.4% at 30 DAS, 21.5%, 14.6% and 5.2% at 60 DAS, respectively over their respective control.

Lesser decrease in photosynthetic characteristics was noted when plants grown with 100 mM NaCl were supplement with 1 mM SO_4^{2-} . The decrease in net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration was restricted to 9.1%, 28.6% and 6.1% at 30 DAS, 15.9%, 36.0% and 13.9% at 60 DAS in Pusa Jai Kisan and 26.8%, 39.3% and 20.2% at 30 DAS, 32.6%, 47.8% and 24.9% at 60 DAS in SS2, respectively with 1 mM SO_4^{2-} plus 100 mM NaCl over their control. However, application of 2 mM SO_4^{2-} to plants receiving 100 mM NaCl completely ameliorated the salinity stress in both the cultivars and enhanced the above

characteristics by 13.5%, 9.9% and 3.5% at 30 DAS, 27.1%, 10.5% and 4.6% at 60 DAS in Pusa Jai Kisan; and 3.3%, 5.4% and 1.6% at 30 DAS, 12.4%, 5.8% and 1.7% at 60 DAS in SS2 over their respective control.

In Pusa Jai Kisan, 100 mM NaCl significantly increased transpiration rate by 41.9% at 30 DAS and 45.8% at 60 DAS, but the increase was higher in SS2, which was 49.0% at 30 DAS and 86.0% at 60 DAS in comparison to control. Application of 1 and 2 mM SO_4^{2-} reduced transpiration rate by 33.6% and 6.6% at 30 DAS, 24.7% and 2.2% at 60 DAS in Pusa Jai Kisan; while in SS2 it was lowered by 28.4% and 7.2% at 30 DAS, 20.6% and 1.4% at 60 DAS in comparison to control.

Supplementation of 1 mM SO_4^{2-} to NaCl-treated plants lowered the transpiration rate but 2 mM SO_4^{2-} completely reversed the adverse effect of NaCl on transpiration rate. In Pusa Jai Kisan, 1 mM SO_4^{2-} reduced the transpiration rate but 2 mM SO_4^{2-} application maximally reduced the transpiration rate by 27.3% at 30 DAS and 20.7% at 60 DAS in comparison to control when applied to NaCl treated plant. In SS2, the application of 1 and 2 mM SO_4^{2-} to NaCl-induced plant caused lesser reduction in transpiration rate compared to Pusa Jai Kisan.

Water-use efficiency and chlorophyll fluorescence decreased significantly in both the cultivars under salinity stress and the maximum decrease was noted in SS2. In Pusa Jai Kisan, water-use efficiency and chlorophyll fluorescence were decreased by 57.3% and 33.9% at 30 DAS, 62.7% and 37.1% at 60 DAS; while in SS2 these traits were decreased by 64.7% and 44.8% at 30 DAS, 77.1% and 50.2% at 60 DAS due to 100 mM NaCl treatment in comparison to control. Application of 1 mM SO_4^{2-} proved beneficial and increased the water-use efficiency and chlorophyll fluorescence by 93.4% and 9.6% at 30 DAS, 73.3% and 10.5% at 60 DAS in Pusa Jai Kisan. In SS2, the above characteristics were increased by 60.1% and 6.9% at 30 DAS, 53.2% and 6.4% at 60 DAS in comparison to control. However, application of 2mM SO_4^{2-} was found inhibitory to water-use efficiency and chlorophyll fluorescence decreasing these by 11.7% and 13.5% at 30 DAS, 21.4% and 30.8% at 60 DAS in Pusa Jai Kisan and 25.5% and 20.5% at 30 DAS, 40.1% and 43.4% at 60 DAS in SS2 over their control.

Application of sulfur to plants grown with 100 mM NaCl ameliorated the NaCl-induced stress. Maximum ameliorative effect was noted with 2 mM SO_4^{2-} in Pusa Jai Kisan than SS2. The increase in water-use efficiency and chlorophyll fluorescence with 2 mM SO_4^{2-} of NaCl-grown plants was 56.2% and 2.3% at 30 DAS, 60.5% and 3.8% at

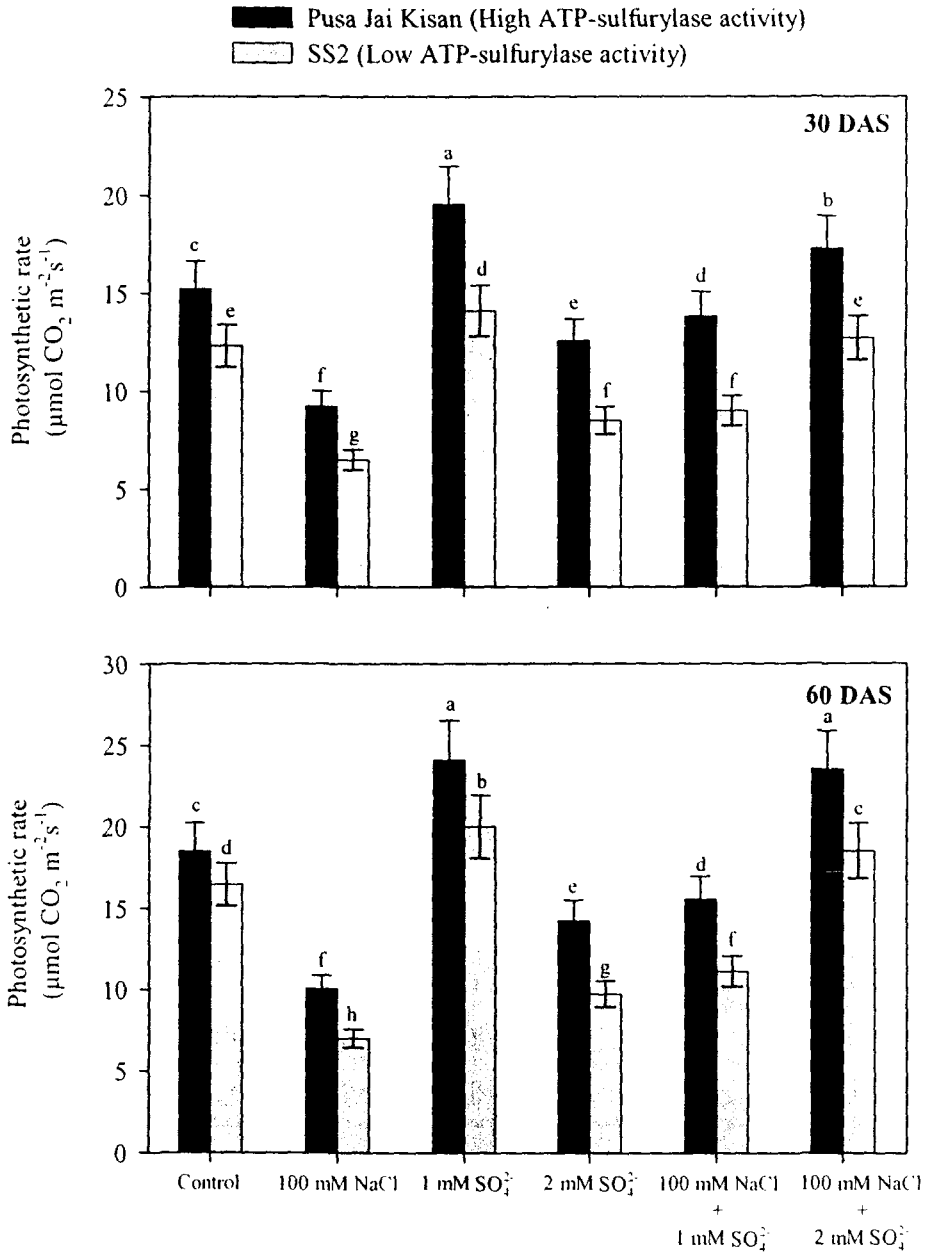


Figure 39. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on photosynthetic rate at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

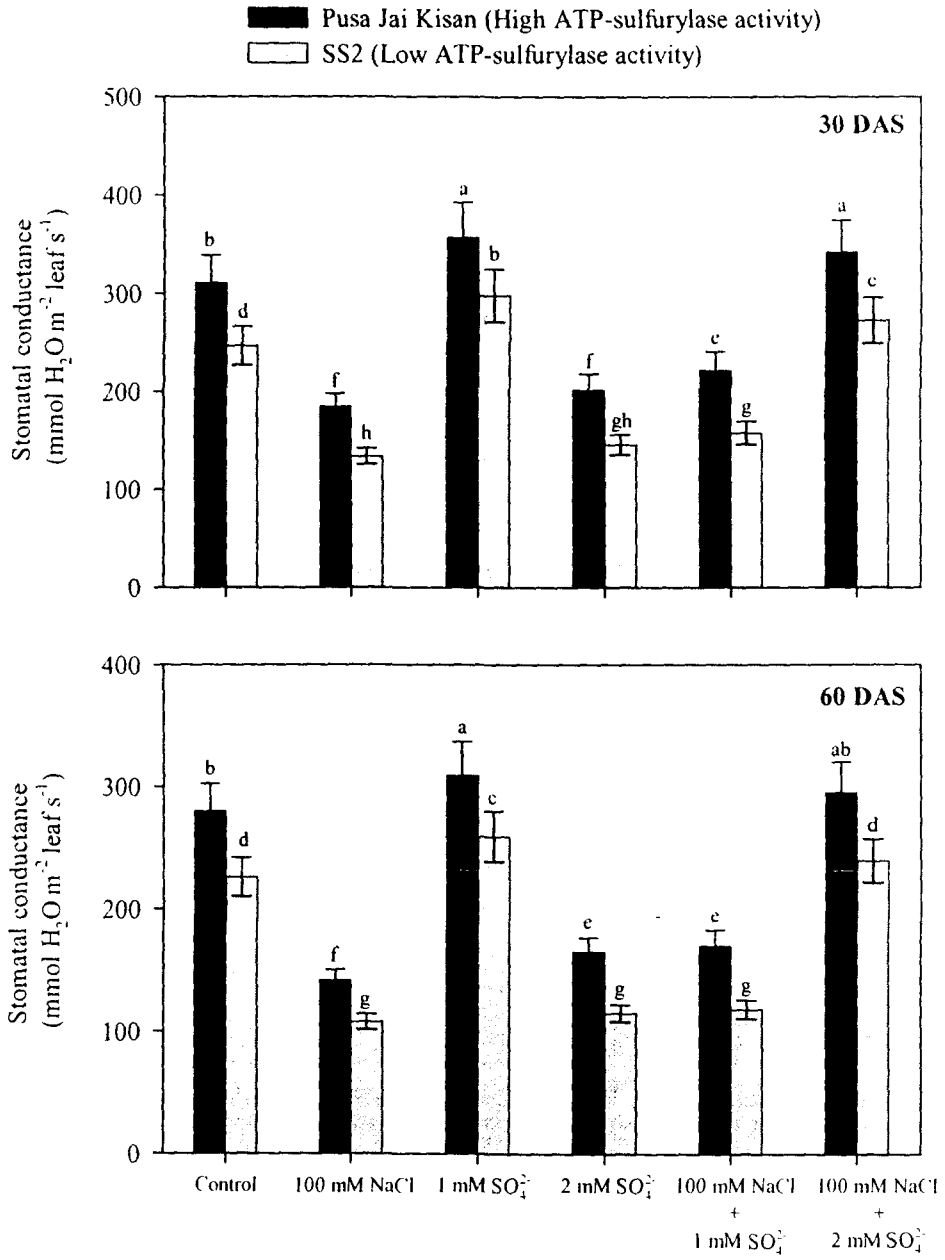


Figure 40. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on stomatal conductance at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

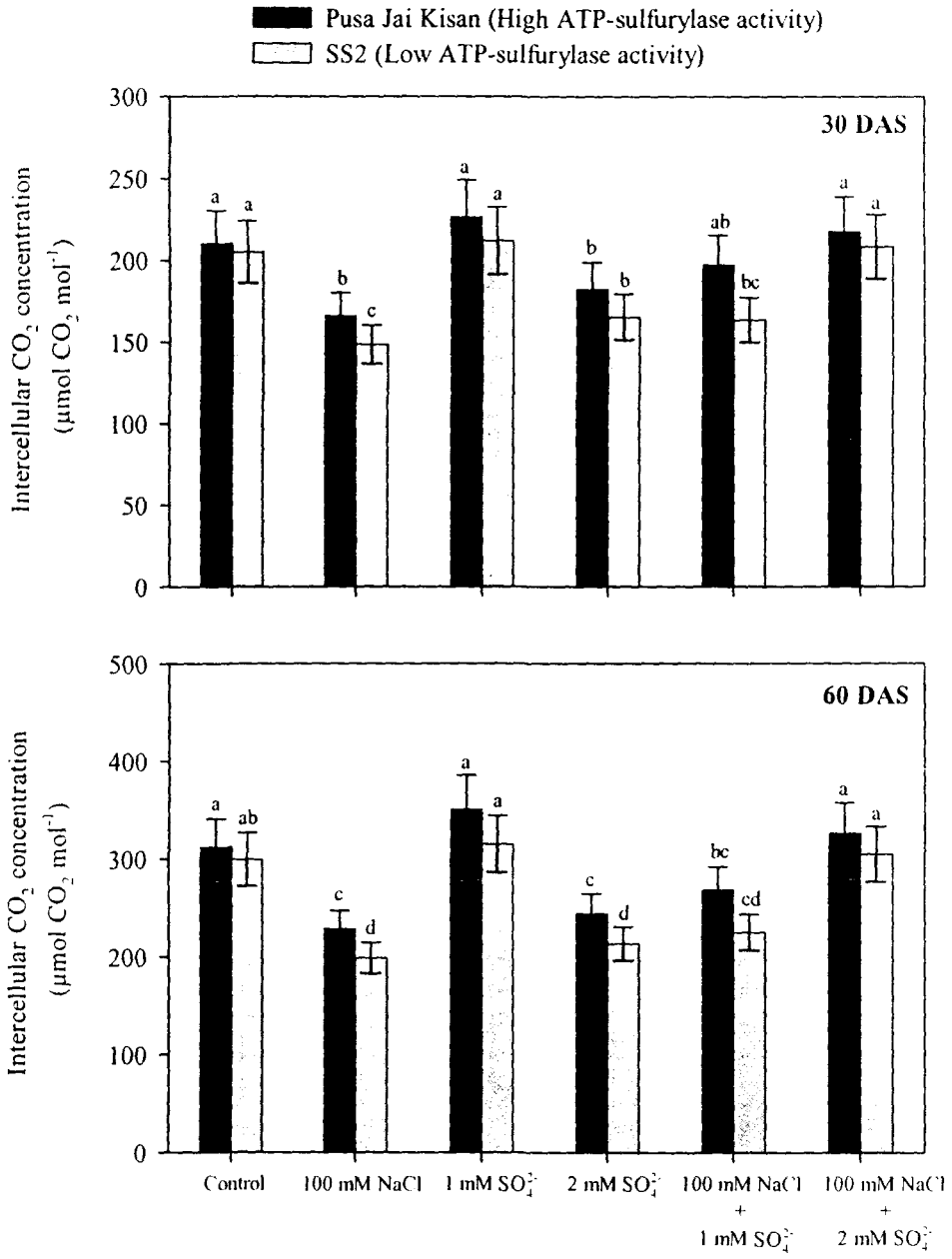


Figure 41. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on intercellular CO_2 concentration at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

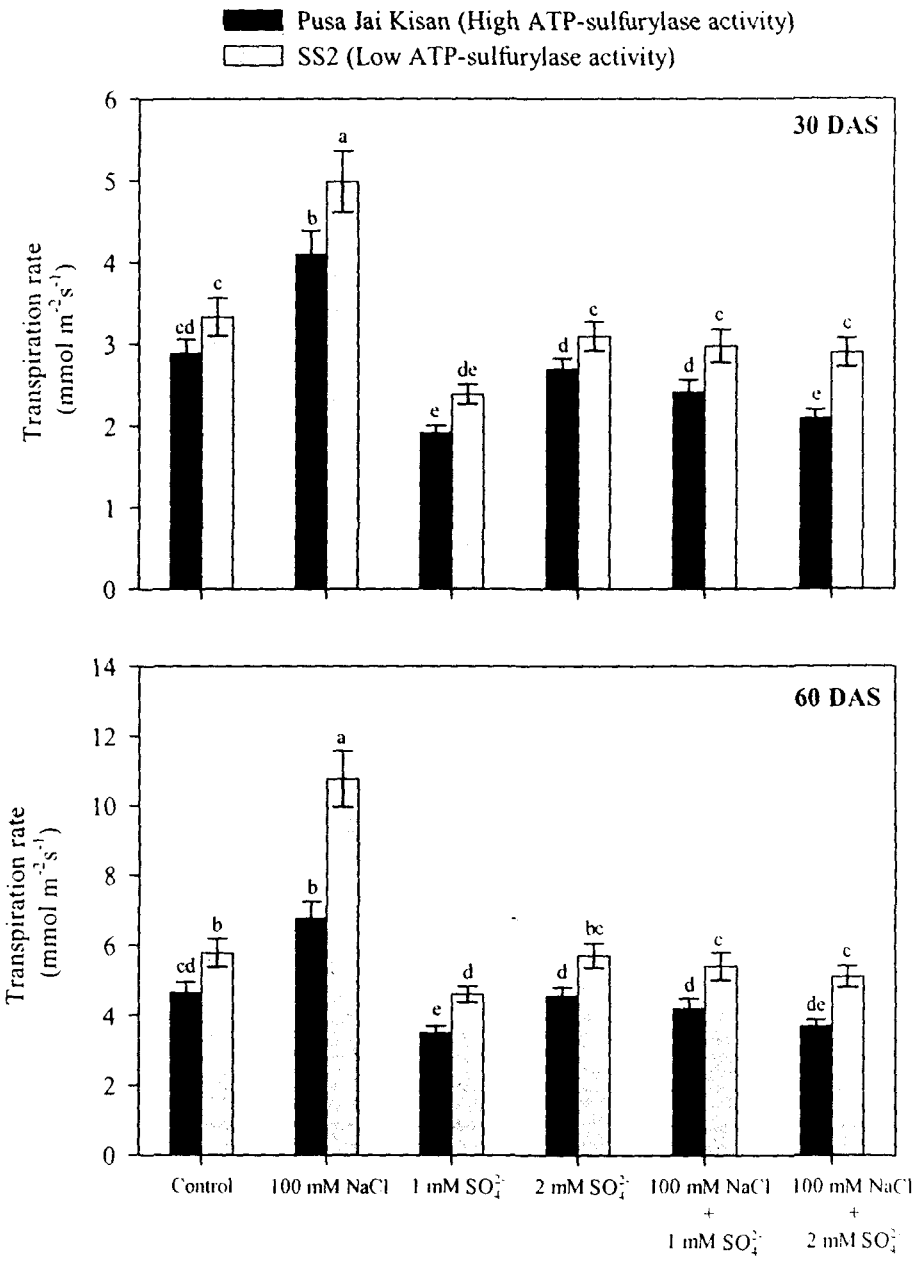


Figure 42. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on transpiration rate at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

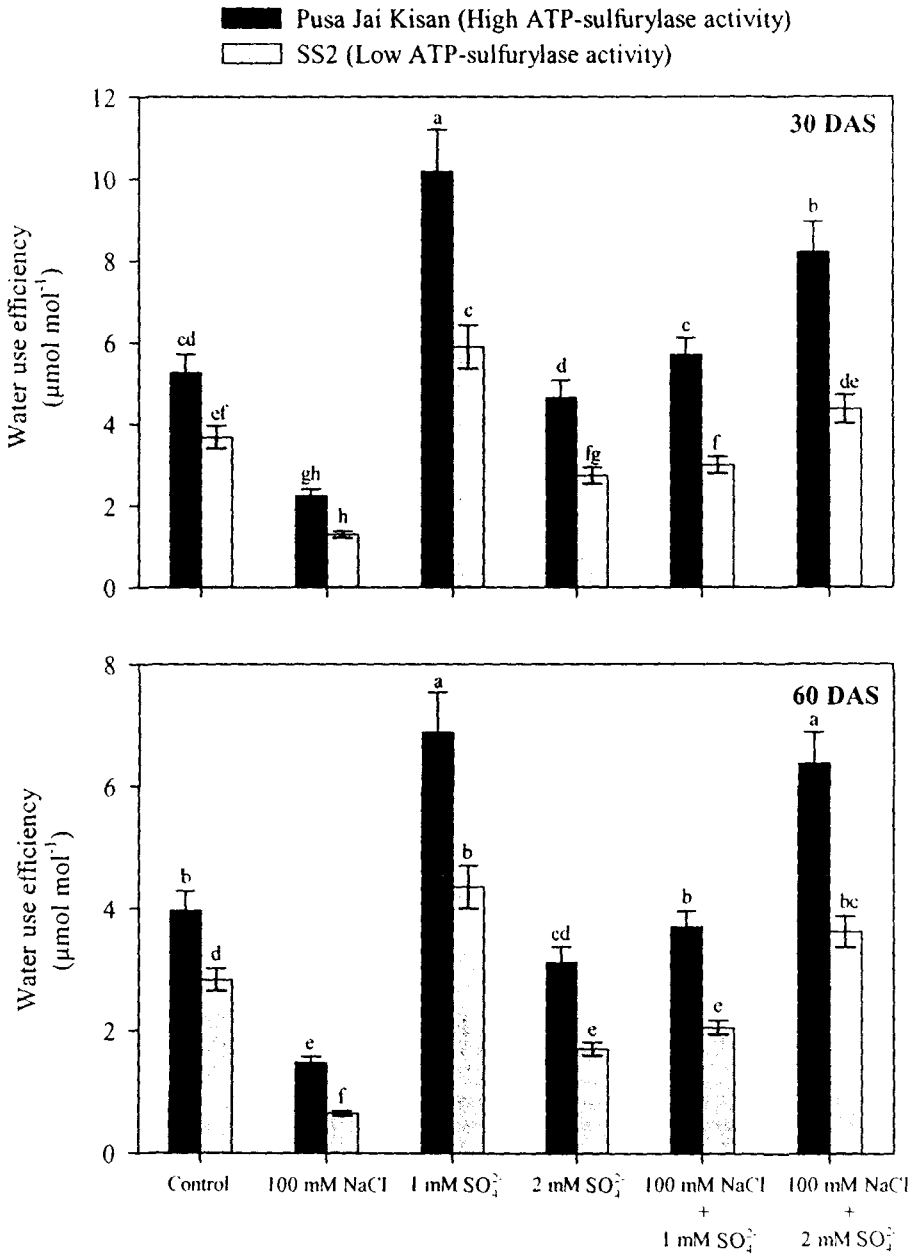


Figure 43. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on water use efficiency at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

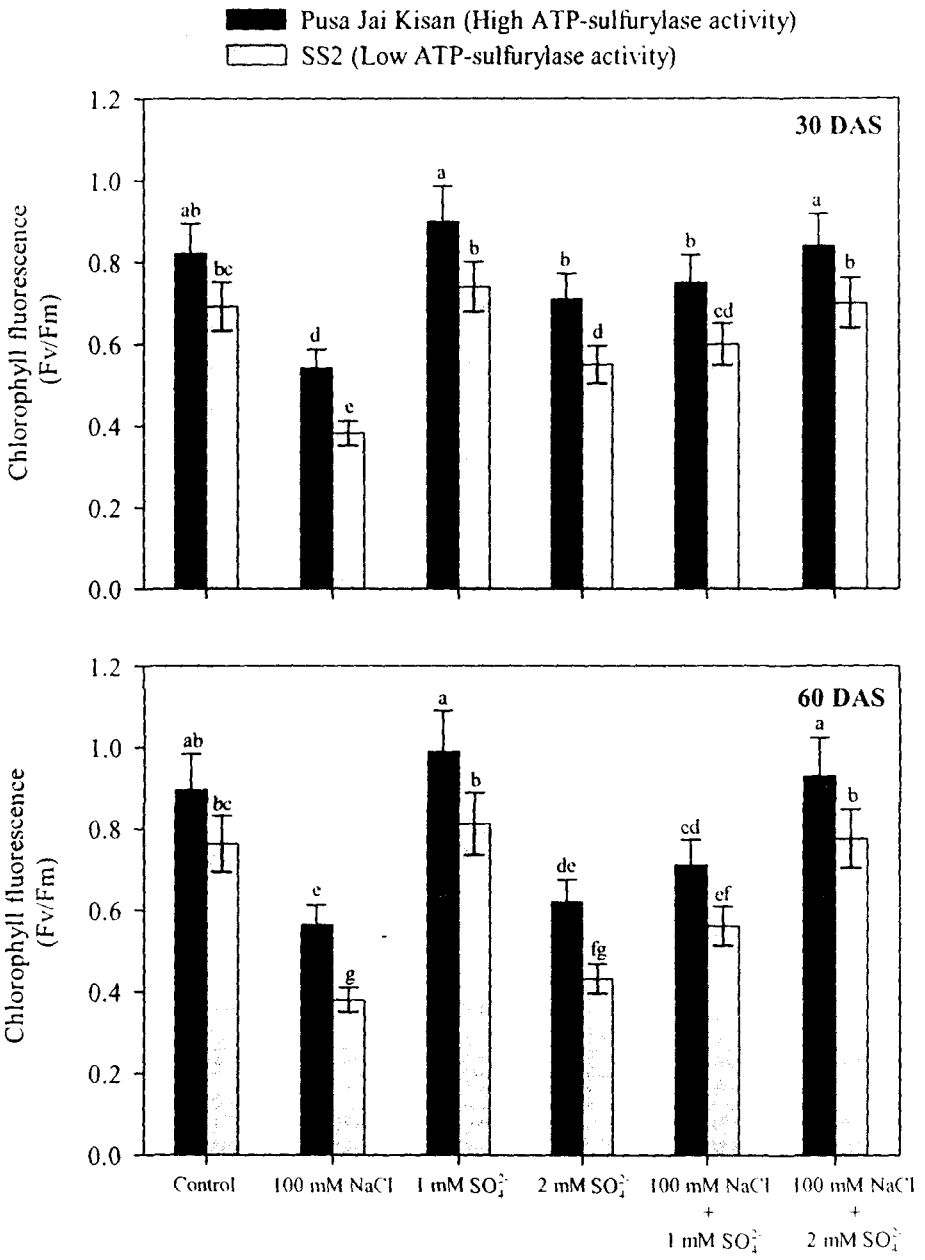


Figure 44. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on chlorophyll fluorescence at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

60 DAS in Pusa Jai Kisan and 18.8% and 1.2% at 30 DAS, 27.5% and 0.9% at 60 DAS in SS2 over the respective control.

4.3.3 Water Relations

NaCl treatment significantly decreased leaf water potential and osmotic potential in both the cultivars and the decrease was maximum in SS2. Sulfur supplementation (1 and 2 mM SO_4^{2-}) to NaCl-treated plants alleviated the adverse effects of NaCl on leaf water potential and osmotic potential (Figures 45-46). The adverse effect was completely overcome by 2 mM SO_4^{2-}, whereas 1 mM SO_4^{2-} application only reduced the severity of NaCl stress (Figures 45-46).

In Pusa Jai Kisan, leaf water potential and osmotic potential were decreased by 71.2% and 97.4% at 30 DAS, 106.1% and 124.2% at 60 DAS with 100 mM NaCl in comparison to control. The cultivar SS2 showed a decrease of 131.3% and 118.3% at 30 DAS, 165.9% and 162.8% at 60 DAS compared to control.

Application of 1 mM SO_4^{2-} increased water potential and osmotic potential in Pusa Jai Kisan by 32.2% and 22.4% at 30 DAS, 46.3% and 29.3% at 60 DAS over their respective control. In SS2, these traits were decreased by 18.8% and 9.8% at 30 DAS, 23.1% and 11.8% at 60 DAS over their respective control. Contrarily, 2 mM SO_4^{2-} application showed lesser increase in water potential and osmotic potential than 1 mM SO_4^{2-}. Hence 1 mM SO_4^{2-} proved to be more beneficial than 2 mM SO_4^{2-} when applied alone.

Addition of 1 mM SO_4^{2-} and 2 mM SO_4^{2-} completely ameliorated the NaCl stress in both the cultivars but maximum amelioration was done by 2 mM SO_4^{2-}. The leaf water potential in Pusa Jai Kisan was increased by 8.5% and 12.2% when 1 mM SO_4^{2-} was given to 100 mM NaCl treated plants. Application of 2mM SO_4^{2-} to NaCl treated plants caused greater benefit and the increase in leaf water potential was 22.0% and 28.1% compared to control at 30 and 60 DAS. In SS2, leaf water potential was increased by 4.7% and 3.3% due to 1 mM SO_4^{2-} and 10.9% and 19.8% due to 2 mM SO_4^{2-} applied to NaCl stressed plants compared to control at 30 and 60 DAS.

Leaf osmotic potential showed an increase of 3.9% and 6.1% due to 1 mM SO_4^{2-} applied to NaCl treated plants compared to control at 30 and 60 DAS, whereas, supplementation of 2 mM $SO4^{2-}$ maximally ameliorated the NaCl stress in both the cultivars.

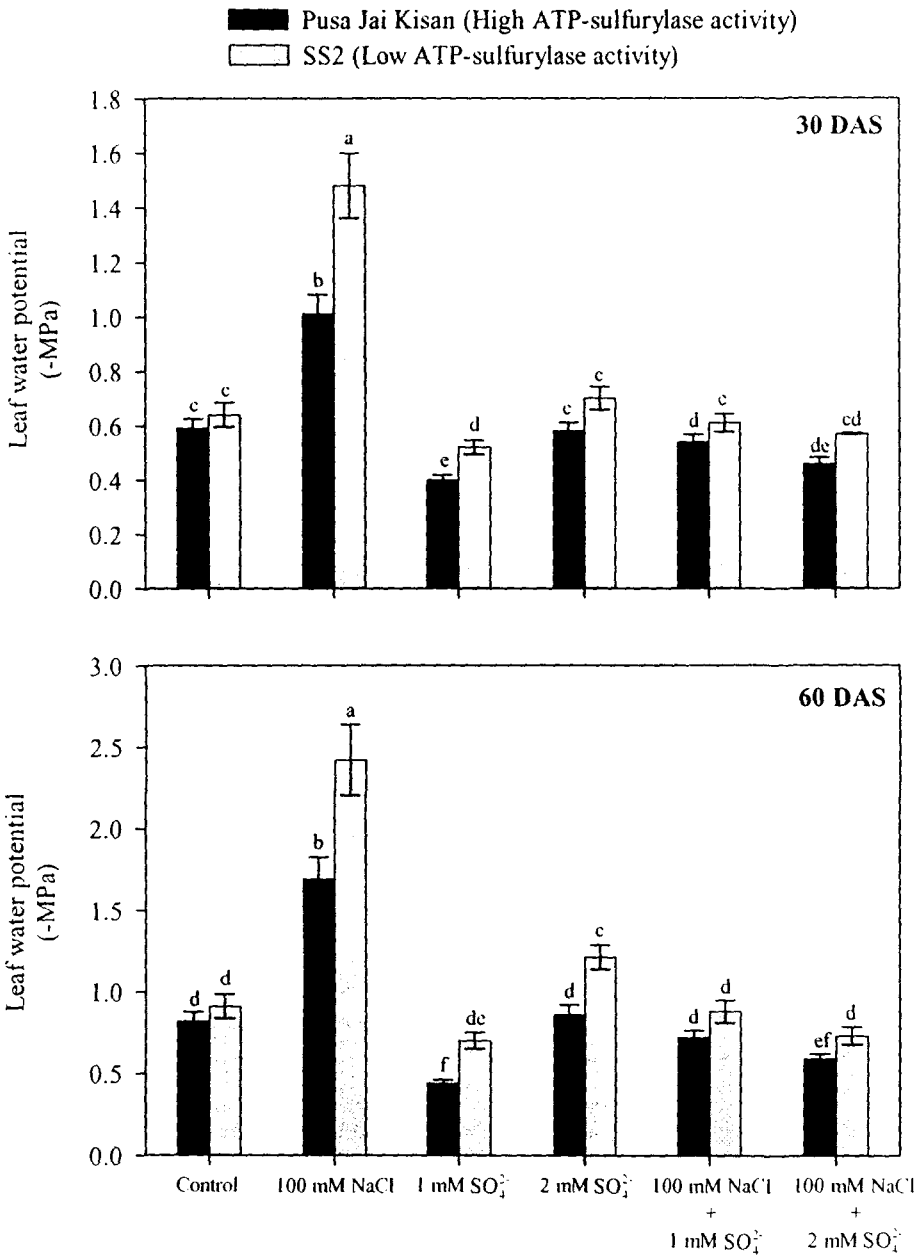


Figure 45. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on leaf water potential at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

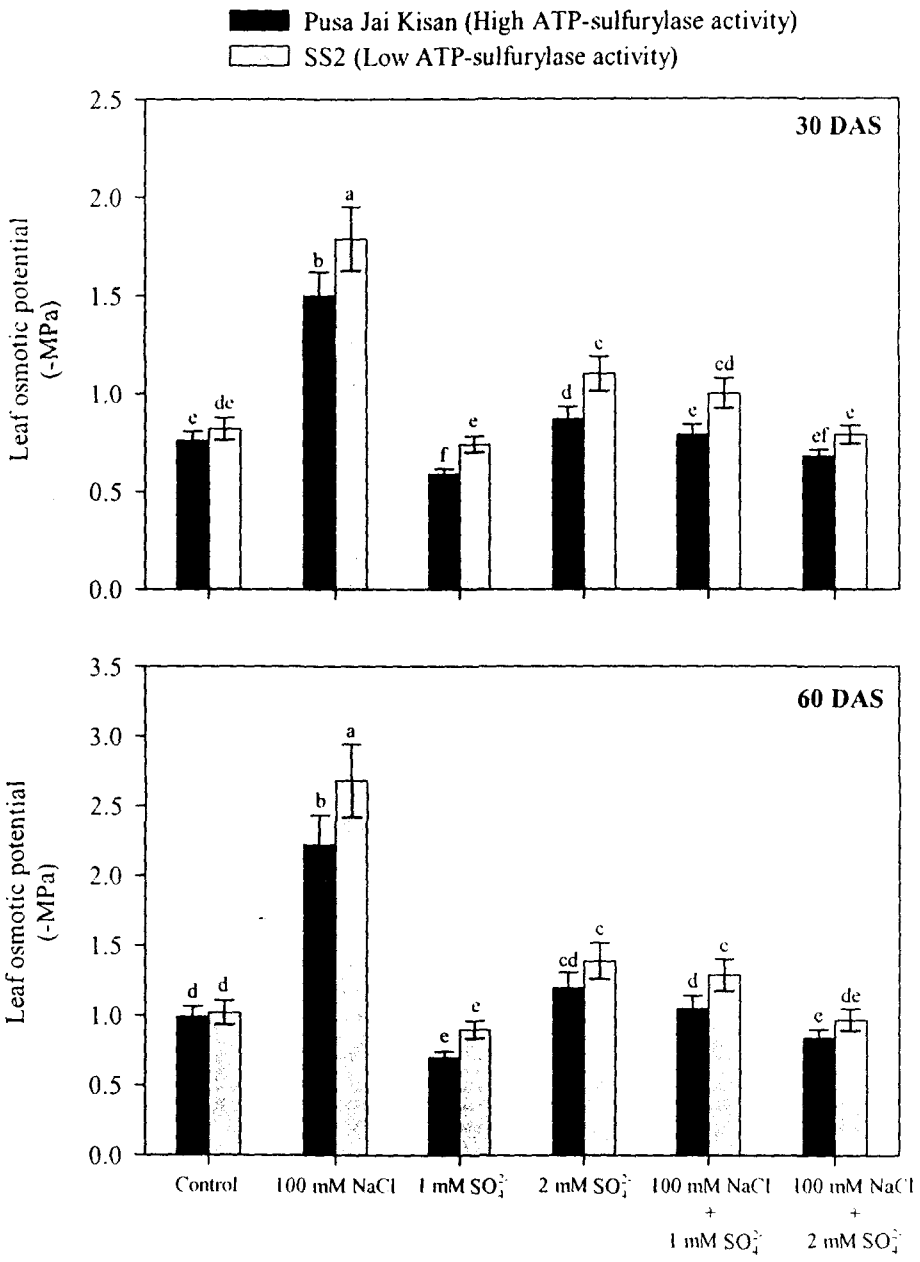


Figure 46. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on leaf osmotic potential at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

4.3.4 Contents of nutrients and ions

4.3.4.1 Nutrients

The content of nutrients (N, P, K and Ca) decreased significantly with NaCl treatment in both the cultivars. The decrease was greater in SS2 than Pusa Jai Kisan. Application of 1 mM SO_4^{2-} alone was more effective in increasing the nutrients content, whereas application of 2 mM SO_4^{2-} resulted in the decrease of the nutrients content compared to the control. The ameliorative effect of 1 mM SO_4^{2-} on NaCl-induced reduction in nutrients content was lesser compared to 2 mM SO_4^{2-} (Figures 47-50).

The decrease in leaf N, P, K and Ca content with 100 mM NaCl was 28.7% 36.7% 35.0% and 37.1% at 30 DAS, 31.0% 49.9% 40.5% and 42.8% at 60 DAS, respectively compared to control. In SS2, the decrease in these nutrients content was 38.5%, 46.0%, 49.3% and 46.1% at 30 DAS and 45.2%, 59.9%, 54.4% and 52.2% at 60 DAS, respectively with respect to control.

Treatment of 1 mM SO_4^{2-} alone increased the content of N, P, K and Ca by 10.7%, 19.5%, 32.0% and 41.9% at 30 DAS, 11.3%, 23.7%, 42.1% and 53.1%, respectively at 60 DAS in Pusa Jai Kisan; while 2 mM SO_4^{2-} application decreased the nutrients content (although less than 100 mM NaCl) by 18.9%, 19.9%, 18.0% and 25.8% at 30 DAS, 24.2%, 39.4%, 24.7% and 30.3% at 60 DAS, respectively compared to control. Similarly, in SS2, application of 1 mM SO_4^{2-} resulted in the increase in N, P, K and Ca content by 9.4%, 12.0%, 24.0% and 28.4% at 30 DAS, 9.5%, 19.3%, 30.0% and 41.2%, respectively at 60 DAS. In contrast, application of 2 mM SO_4^{2-} decreased these nutrients content by 28.7%, 32.7%, 33.3% and 25.5% at 30 DAS, 34.8%, 50.0%, 31.3% and 40.4%, respectively at 60 DAS in comparison to their respective control.

The supplementation of SO_4^{2-} (1 or 2 mM) lowered the 100 mM NaCl-caused reductions in the content of N, P K and Ca in both the cultivars. However, the supplementation of 2 mM SO_4^{2-} completely alleviated the adverse effect of 100 mM NaCl.

In Pusa Jai Kisan, the application of 2 mM SO_4^{2-} to 100 mM NaCl-treated plants completely ameliorated the reductions and increased the content of above nutrients by 7.4%, 13.1%, 27.0% and 21.8% at 30 DAS, 8.3%, 18.5%, 36.8% and 39.8% at 60 DAS, respectively, over the control. In SS2, the application of 2 mM SO_4^{2-} ameliorated the 100 mM NaCl-caused reductions in the content of nutrients by 4.2%,

6.7%, 18.7% and 7.8% at 30 DAS, 5.7%, 16.8%, 23.1% and 17.7% at 60 DAS, respectively with respect to control.

4.3.4.2 Ions

Salinity stress increased Na^+ content in both root and leaf in both the cultivars at both the growth stages. Application of 1 mM SO_4^{2-} to the plants reduced the Na^+ content in both root and leaf. Sulfur application was found to alleviate the NaCl effects. Application of 2 mM SO_4^{2-} showed greater amelioration than 1 mM SO_4^{2-} under NaCl stress (Figures 51-54).

In Pusa Jai Kisan, the increase in Na^+ content in leaf and root due to 100 mM NaCl was 34.0% and 35.7% at 30 DAS and 39.5% and 30.6% at 60 DAS in comparison to control. However, in SS2, the content of Na^+ in leaf and root due to 100 mM NaCl was increased by 35.3%, and 54.3% at 30 DAS and 50.9% and 36.6% at 60 DAS over the control.

Application of 1 mM SO_4^{2-} reduced the leaf and root Na^+ content equally of about 27.0% in Pusa Jai Kisan at both the stages. However, 2 mM SO_4^{2-} application resulted in the decrease of root Na^+ content but leaf Na^+ content increased in both the cultivars. The decrease in root Na^+ content was 3.2% at 30 DAS and 2.6% at 60 DAS over the control. Leaf Na^+ content was increased by 2.0% at 30 DAS and 18.5% at 60 DAS in comparison to control in Pusa Jai Kisan. The cultivar SS2 showed decrease in leaf and root Na^+ content by 22.5% and 31.0% at 30 DAS, 17.8 and 27.6% at 60 DAS with 1 mM SO_4^{2-} application. Treatment of 2 mM SO_4^{2-} reduced root Na^+ content by 6.0% at 30 DAS and 8.4% at 60 DAS in comparison to control, but the leaf Na^+ content was increased by 4.9% at 30 DAS and 29.9% at 60 DAS over control.

Supplementation of 1 mM SO_4^{2-} to NaCl treated plants completely reversed the increase in Na^+ content in both leaf and root through decreasing it by 9.3% and 8.1% at 30 DAS, 6.7% and 10.9% at 60 DAS compared to control in Pusa Jai Kisan. Application of 2 mM SO_4^{2-} to NaCl-stressed plants caused greater amelioration by decreasing the content of leaf and root Na^+ by 16.1% and 21.0% at 30 DAS, 15.0% and 18.1% at 60 DAS. In SS2, content of Na^+ in leaf and root of NaCl treated plant was reduced by 5.8% and 11.7% at 30 DAS, 4.0% and 14.0% at 60 DAS compared to control with the application of 1 mM SO_4^{2-}. The application of 2 mM SO_4^{2-} completely ameliorated the effect of NaCl on leaf and root Na^+ content by reducing these to 13.4% and 24.8% at 30 DAS, 10.2%, and 21.7% at 60 DAS compared to control.

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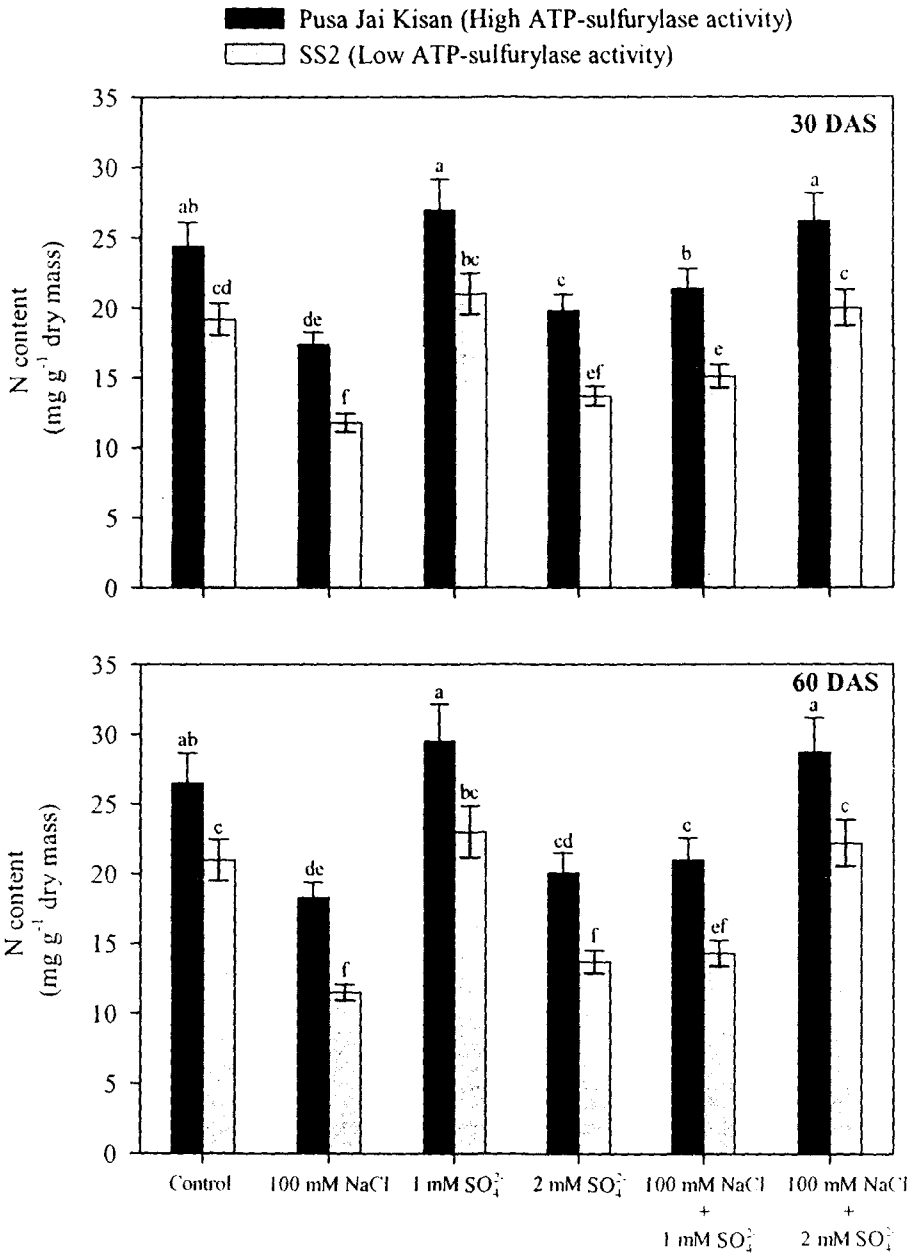

Figure 47. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on N content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

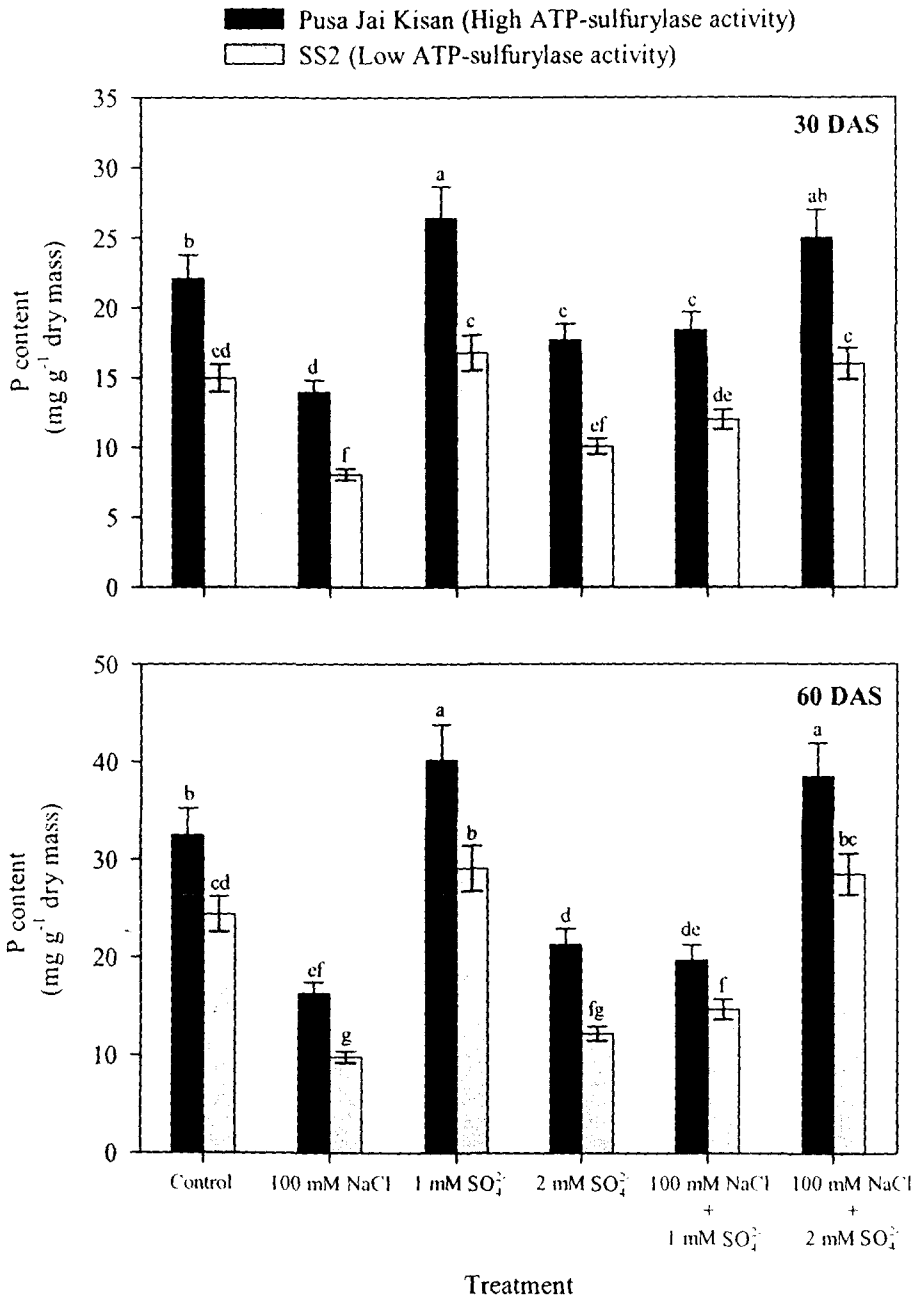

Figure 48. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on P content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

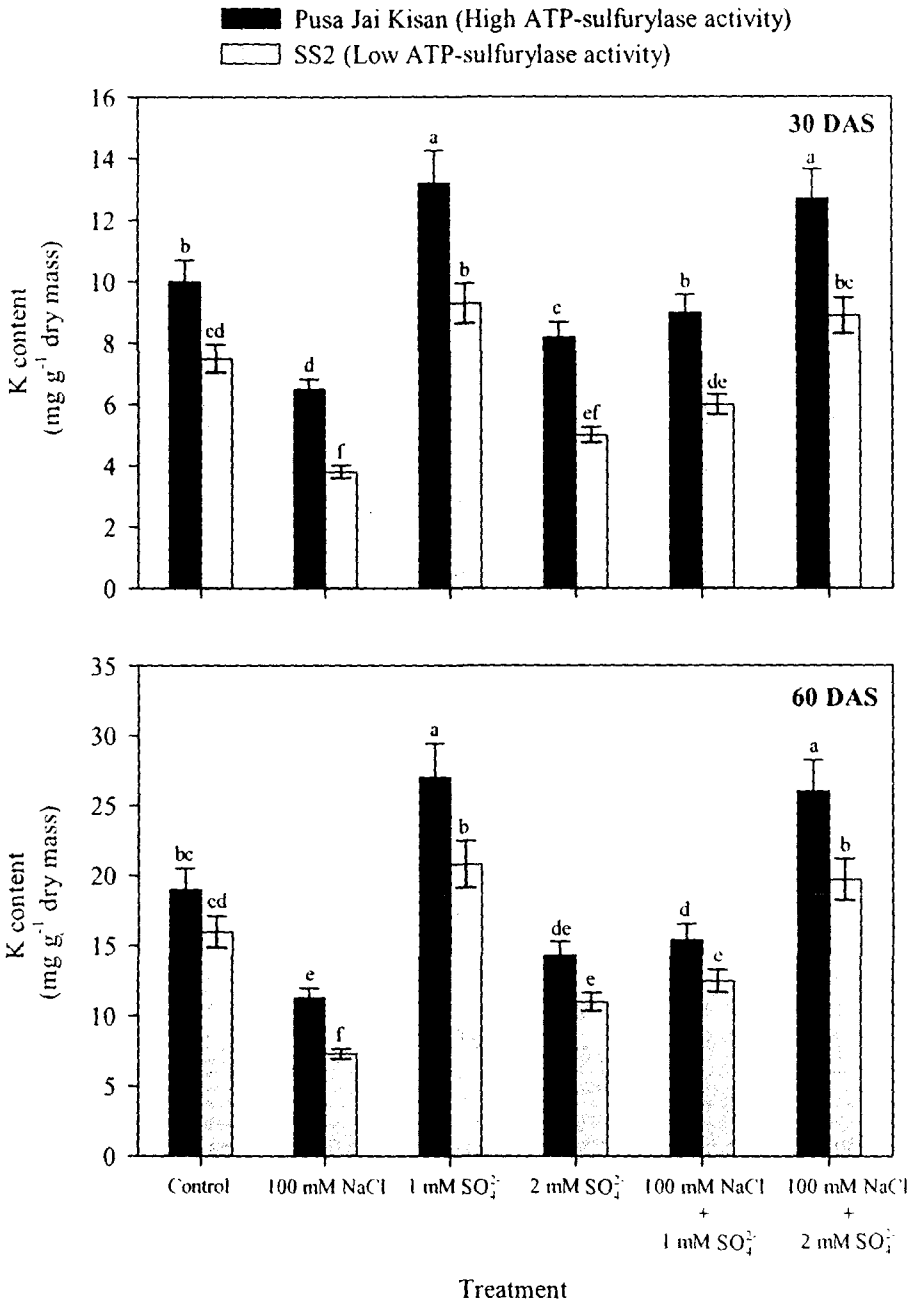

Figure 49. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on K content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

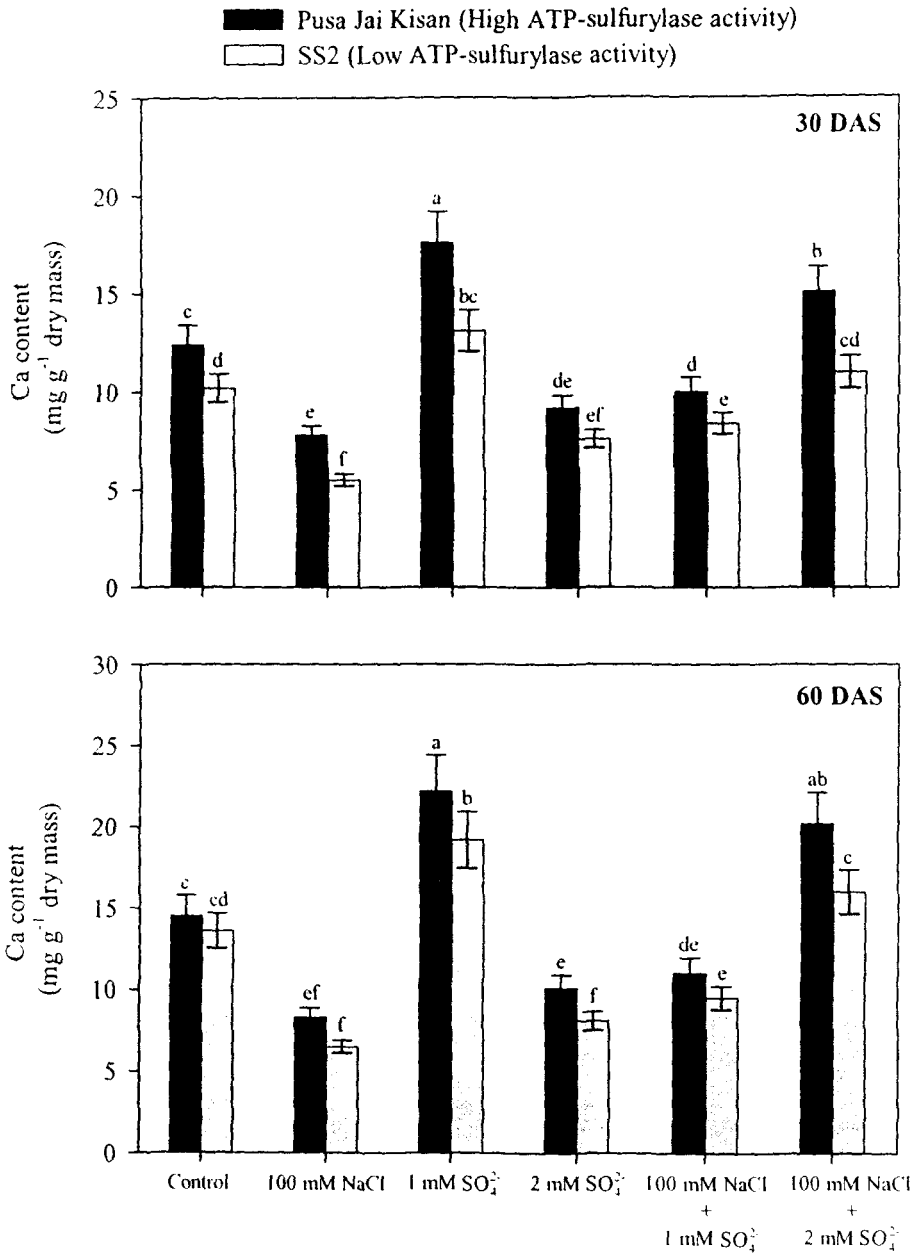

Figure 50. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on Ca content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

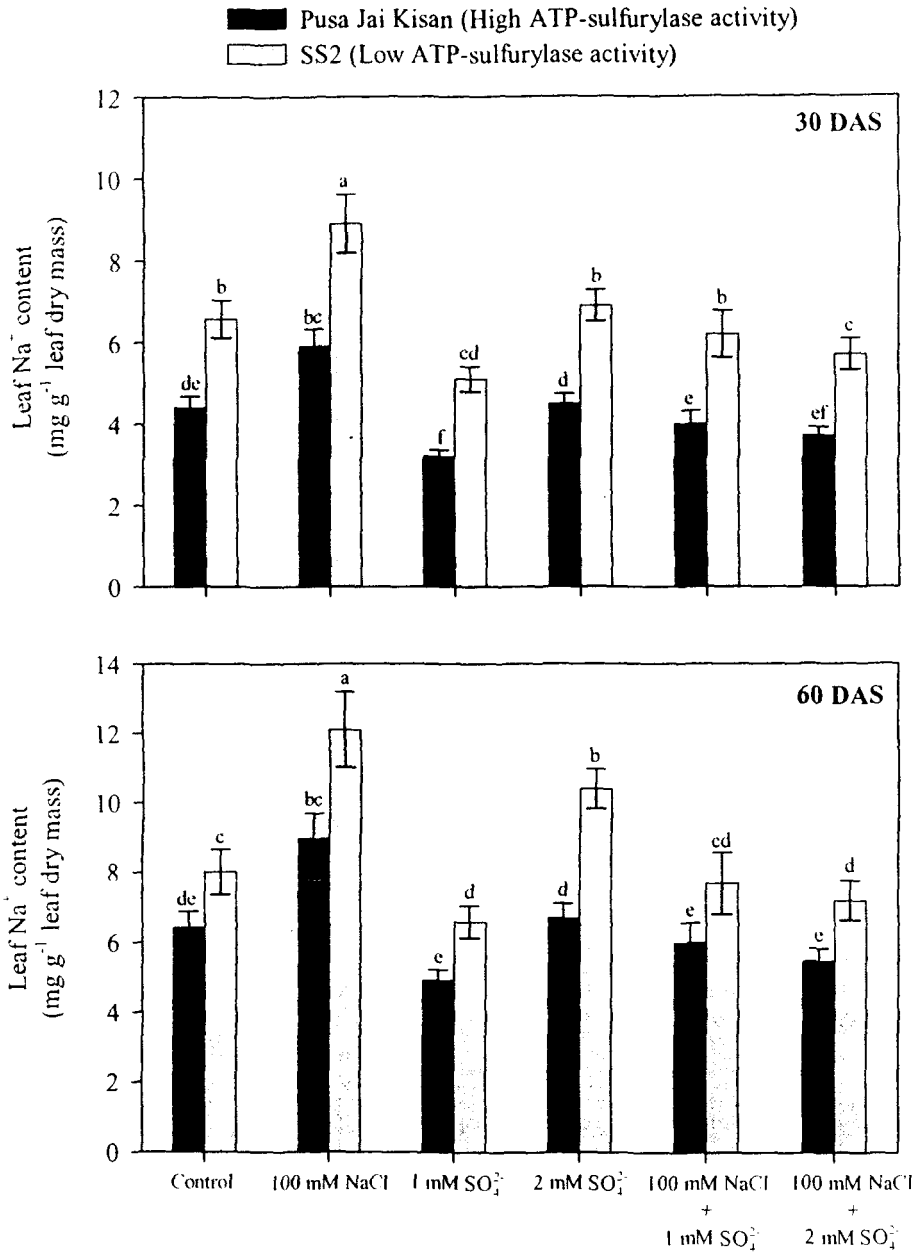

Figure 51. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on leaf Na^+ content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

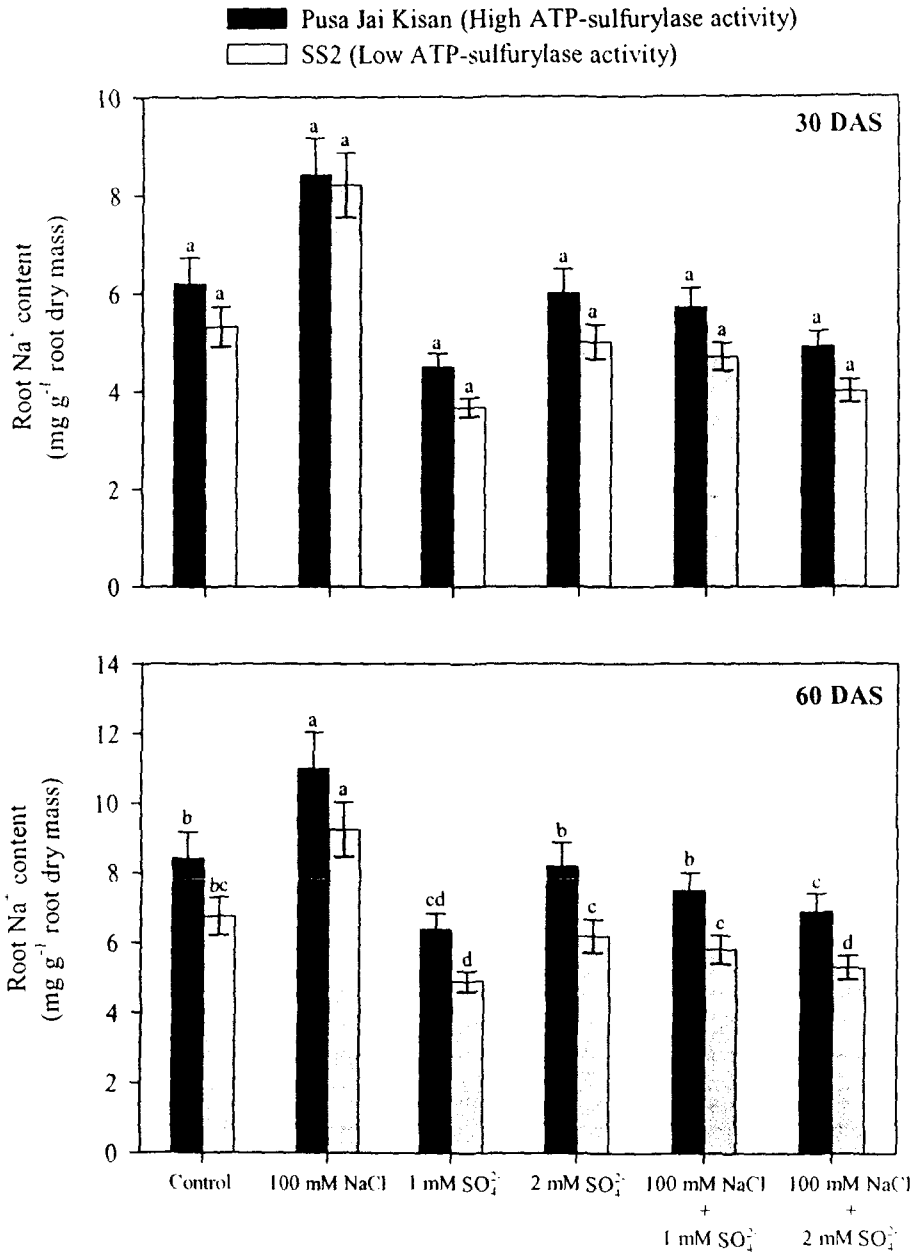

Figure 52. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on root Na^+ content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

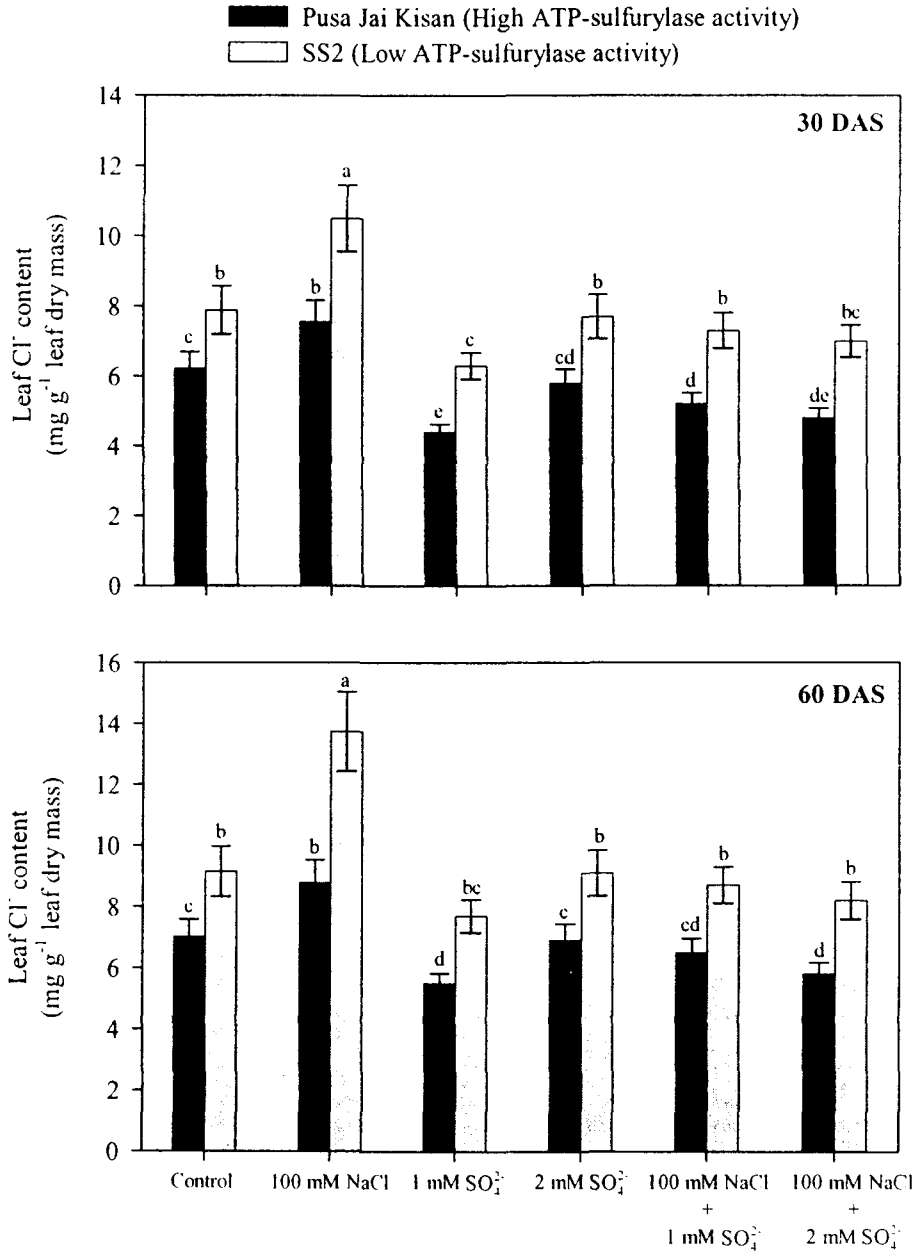

Figure 53. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on leaf Cl^- content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

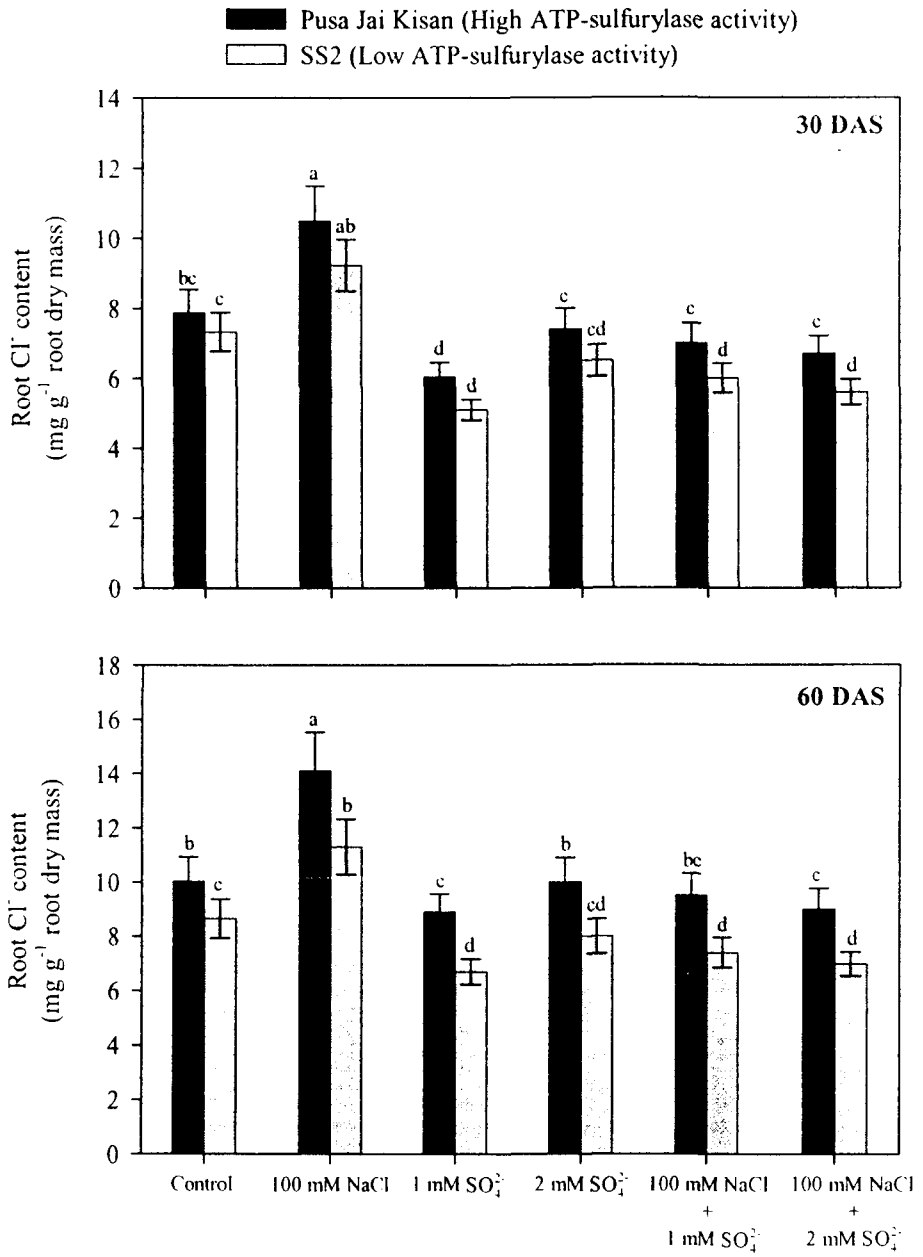

Figure 54. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on root Cl^- content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

NaCl treatment significantly increased the Cl^- content in root and leaf in both the cultivars at both the growth stages. Application of sulfur either alone or in combination with 100 mM NaCl was effective in reducing the increase in Cl^-.

In Pusa Jai Kisan, 100 mM NaCl increased root and leaf Cl^- content by 33.4% and 21.5% at 30 DAS and 40.6% and 25.1% at 60 DAS over the control, while in SS2 the content was increased by 26.1% and 33.1% at 30 DAS, 30.7% and 50.2% at 60 DAS compared to control.

Application of 1 mM SO_4^{2-} alone decreased root and leaf Cl^- content by 23.3% and 29.3% at 30 DAS, 11.3% and 21.7% at 60 DAS in Pusa Jai Kisan. Application of 2 mM SO_4^{2-} resulted in lesser decrease in root and leaf Cl^- content than 1 mM SO_4^{2-}. Similar response of sulfur application was noted for the cultivar SS2.

Supplementation of sulfur (1 mM SO_4^{2-} and 2 mM SO_4^{2-}) completely ameliorated the NaCl stress effects and decreased Cl^- content in root and leaf in both the cultivars. The root Cl^- content was reduced to a higher extent in SS2 than in Pusa Jai Kisan at both the growth stages, while leaf Cl^- content was reduced to a higher extent in Pusa Jai Kisan than SS2. Application of 2 mM SO_4^{2-} caused greater reversal of the adverse effect of NaCl than 1 mM NaCl.

4.3.5 Oxidative stress

NaCl treatment caused oxidative stress in plants in terms of increase in the TBARS and H_2O_2 content, electrolyte leakage, membrane stability index and relative salt injury. Significant increase in the TBARS and H_2O_2 content and electrolyte leakage was observed in both the cultivars and the extent of increase was greater in SS2 than Pusa Jai Kisan. Supplementation of 2 mM SO_4^{2-} maximally reduced the increase in the TBARS and H_2O_2 content and electrolyte leakage caused by NaCl treatment in both the cultivars at both the growth stages (Figures 55-59).

Application of 1 mM SO_4^{2-} alone significantly lowered the contents of TBARS and H_2O_2 and electrolyte leakage in Pusa Jai Kisan and was more effective than 2 mM SO_4^{2-} application. This trend was also noted for SS2.

Salinity stress significantly decreased the membrane stability index in both the cultivars but the decrease was higher in SS2 than Pusa Jai Kisan. The application of 2 mM SO_4^{2-} alone also reduced the membrane stability index but the reduction was less than 100 mM NaCl compared to control. Application of 1 mM SO_4^{2-} proved to be beneficial to Pusa Jai Kisan and increased the membrane stability index by 26.6% at 30

DAS and 34.1% at 60 DAS over the control. In SS2, the increase in membrane stability index due to 1 mM SO_4^{2-} application was 19.4% at 30 DAS and 26.1% at 60 DAS compared to control. Contrarily, in Pusa Jai Kisan, the application of 2 mM SO_4^{2-} decreased the membrane stability index by 10.0% at 30 DAS and 12.1% at 60 DAS compared to control. In SS2, it was decreased by 27.2% at 30 DAS and 37.9% at 60 DAS compared to control.

Supplementation of 2 mM SO_4^{2-} to NaCl-treated plants maximally ameliorated the effect of NaCl on membrane stability index and increased it by 13.9% at 30 DAS and 29.6% at 60 DAS in Pusa Jai Kisan over the control. In SS2, 2 mM SO_4^{2-} application increased it by 10.4% at 30 DAS and 17.1% at 60 DAS compared to control. The application of 1 mM SO_4^{2-} also ameliorated the NaCl effects on membrane stability index to some extent in both the cultivars.

Relative salt injury increased significantly under NaCl treatment and was greater in SS2 than Pusa Jai Kisan. Significant increase in relative salt injury in Pusa Jai Kisan due to 100 mM NaCl was 31.2% at 30 DAS and 57.0% at 60 DAS in comparison to control. In SS2, the increase in relative salt injury due to 100 mM NaCl was 74.2% at 30 DAS and 108.9% at 60 DAS in comparison to control. In Pusa Jai Kisan, the application of 2 mM SO_4^{2-} increased the relative salt injury by 16.0% at 30 DAS and 25.0% at 60 DAS compared to control, whereas in SS2, it was increased by 19.4% at 30 DAS and 33.3% at 60 DAS compared to control. Contrarily, 1 mM SO_4^{2-} reduced the relative salt injury by 28.0% at 30 DAS and 37.5% at 60 DAS in Pusa Jai Kisan and 19.4% at 30 DAS and 28.9% at 60 DAS in SS2 compared to control.

Application of 2 mM SO_4^{2-} to NaCl treated plant maximally ameliorated the effect of NaCl on relative salt injury and was more effective than the application of 1 mM SO_4^{2-} to NaCl-treated plants.

4.3.6 Enzymatic and Non-Enzymatic Antioxidants

4.3.6.1 Enzymatic antioxidants

The response of antioxidant enzymes to NaCl was significant in both the cultivars at both the growth stages. In general, S application (1 mM SO_4^{2-}) alone maximally increased the activity of CAT, APX and GR, whereas its application helped to reduce the increase in SOD activity (Figures 60-63).

The activity of SOD was greater in SS2 than Pusa Jai Kisan under NaCl treatment. SOD activity was increased by 70.2% and 82.6% in Pusa Jai Kisan and

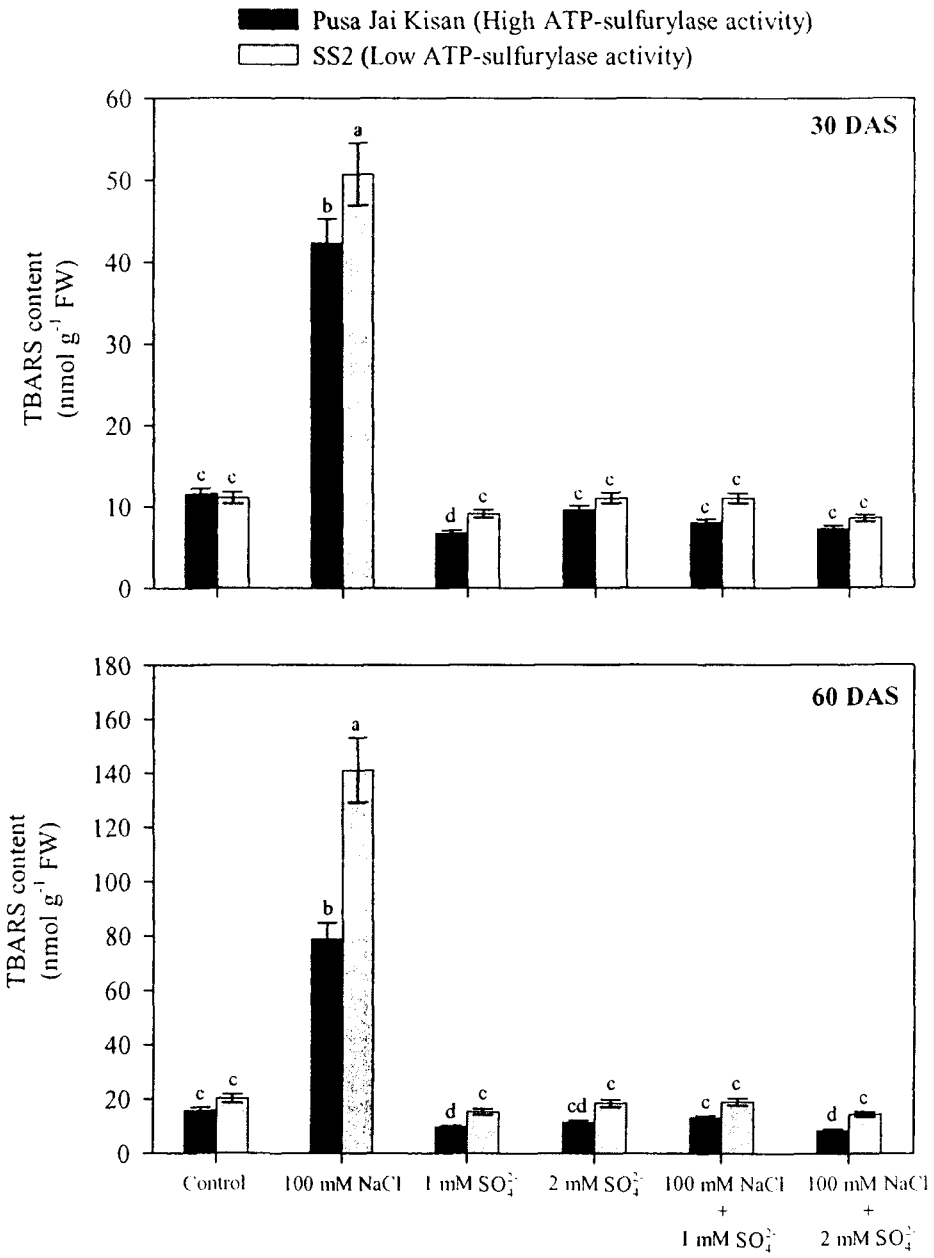

Figure 55. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on TBARS content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

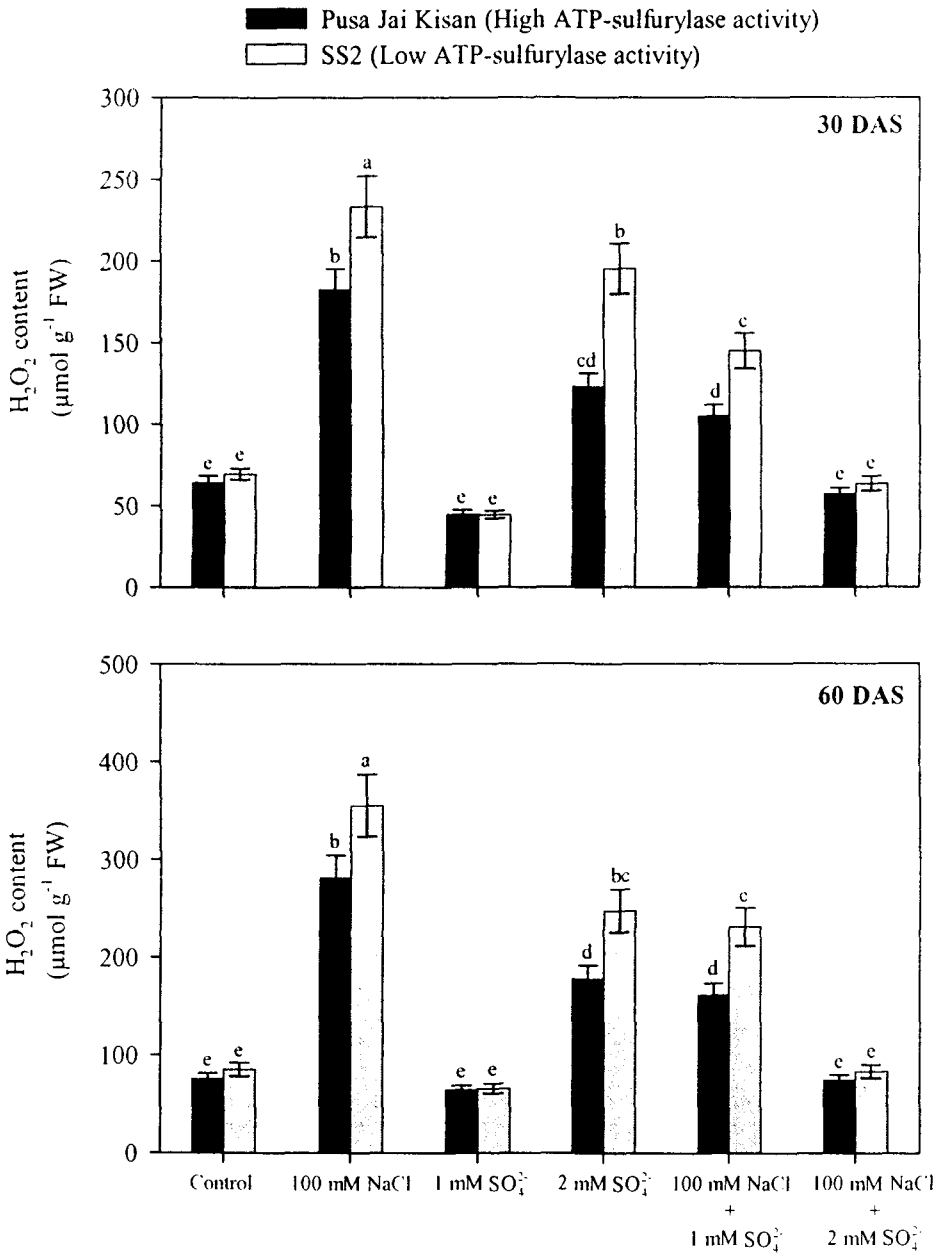

Figure 56. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on H_2O_2 content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

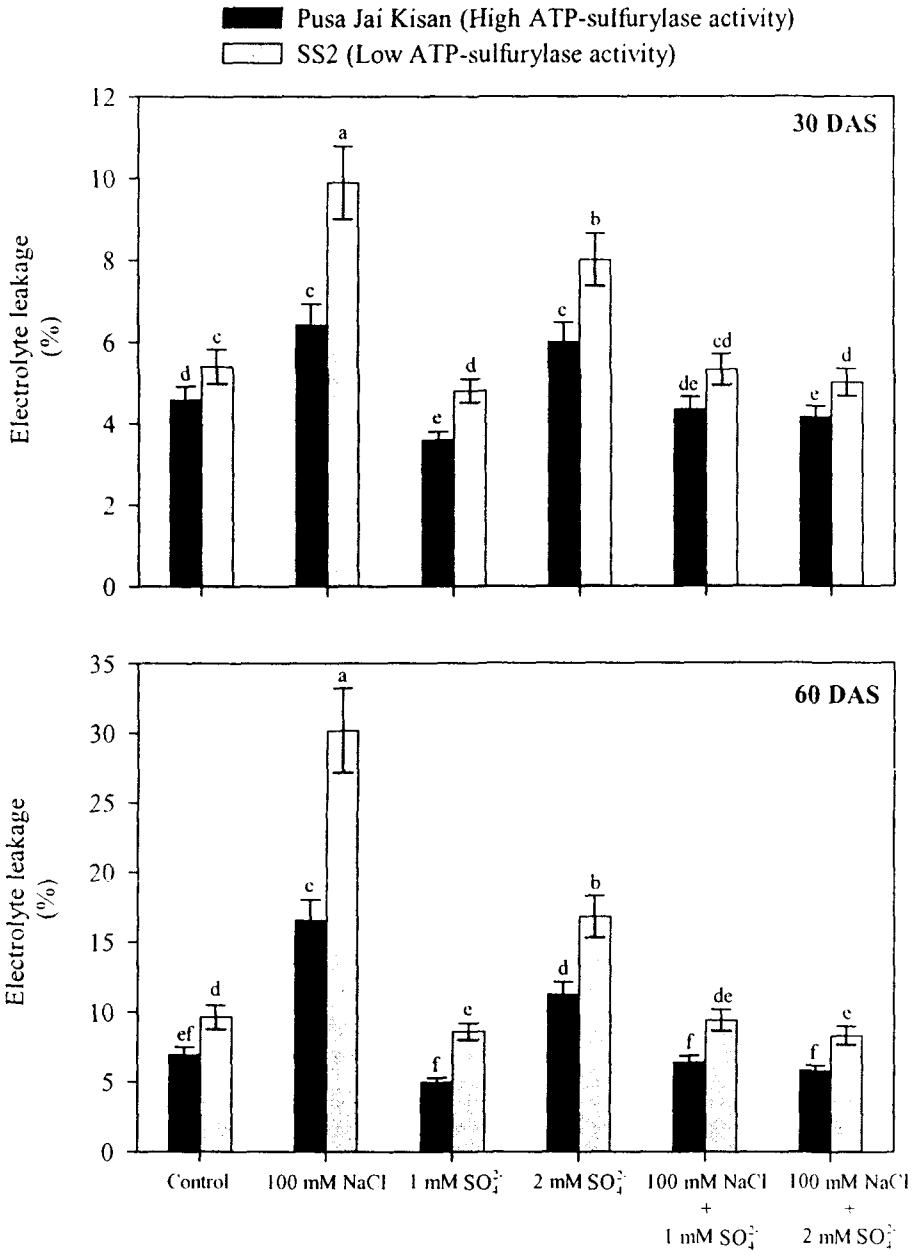

Figure 57. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on electrolyte leakage at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

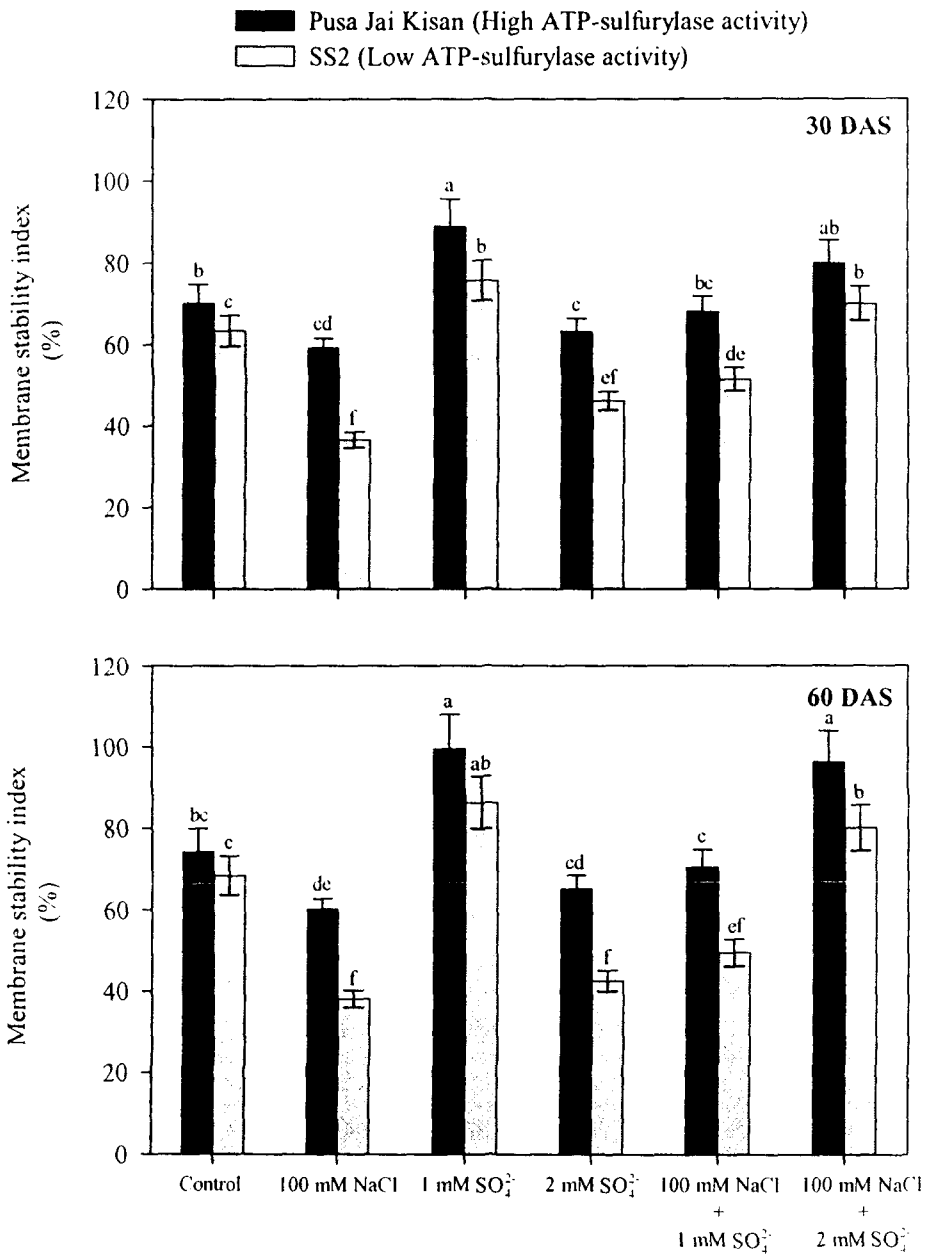

Figure 58. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on membrane stability index at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

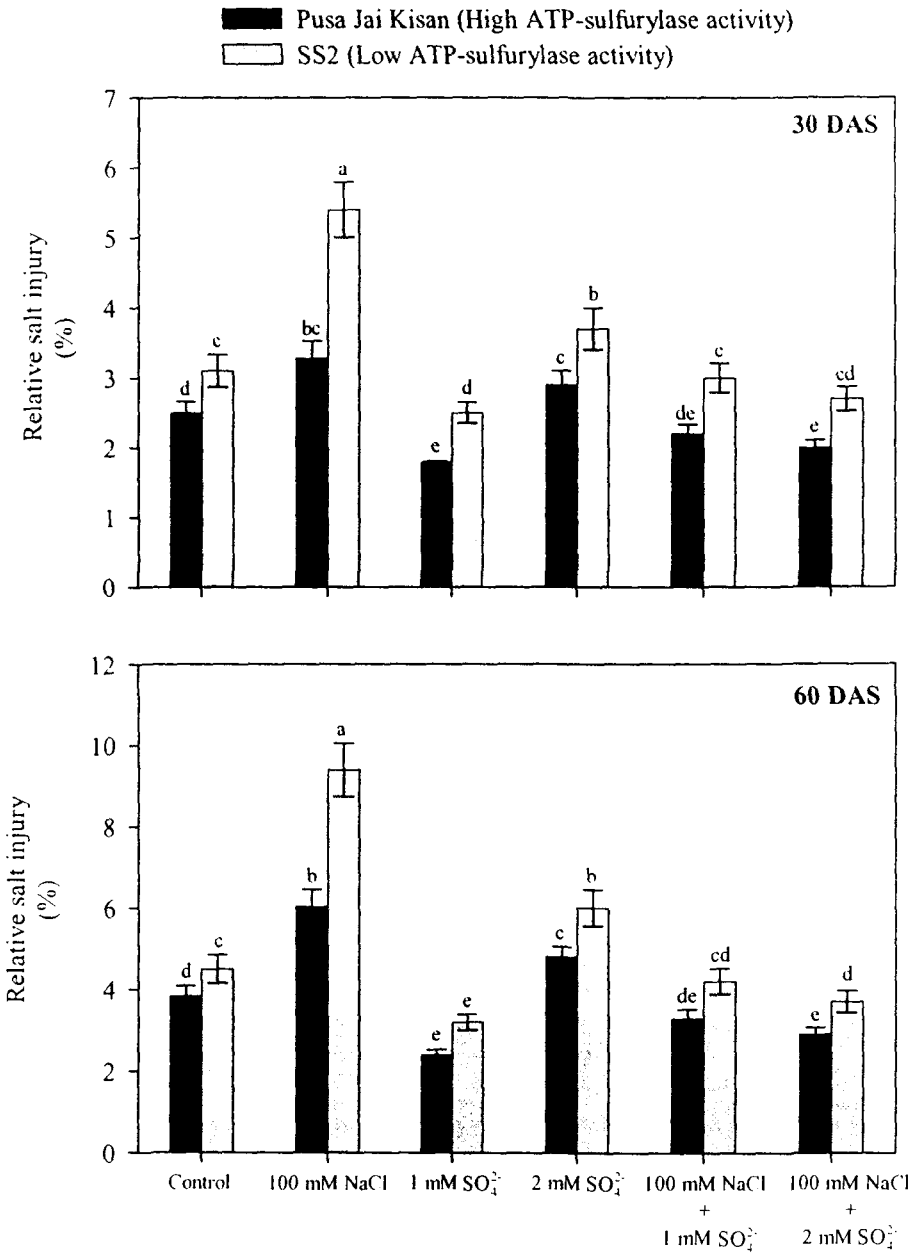

Figure 59. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on relative salt injury at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

105.1% and 112.3% in SS2 at 30 and 60 DAS, respectively due to 100 mM NaCl treatment compared to control.

Application of sulfur as 1mM SO_4^{2-} reduced the SOD activity by 63.5% and 68.4% in Pusa Jai Kisan and 29.7% and 34.8% in SS2 at 30 and 60 DAS in comparison to control. However, 2 mM SO_4^{2-} caused lesser reduction in SOD activity in both the cultivars. The above results indicate that 1 mM SO_4^{2-} is more efficient in reducing SOD activity than 2 mM SO_4^{2-}.

However, on combined application of sulfur and NaCl, 2 mM SO_4^{2-} to NaCl-stressed plants reduced the SOD activity by 40.4% and 46.5% in Pusa Jai Kisan and 22.6% and 31.0% in SS2 at 30 and 60 DAS compared to control; while 1 mM SO_4^{2-} application reduced it by 25.0% and 35.1% in Pusa Jai Kisan and 11.4% and 22.9% in SS2 at 30 and 60 DAS compared to control.

The activity of CAT, APX and GR increased under NaCl at 30 and 60 DAS in both the cultivars. Significant increase in the activity of these enzymes due to 100 mM NaCl was 80.1%, 227.0% and 61.1% at 30 DAS, 128.7%, 242.6% and 164.3% at 60 DAS, respectively in comparison to their respective control in Pusa Jai Kisan. Application of 1 mM SO_4^{2-} alone increased the activity of these enzymes by 120.6%, 297.8% and 155.6% at 30 DAS, 186.5%, 317.6% and 226.0% at 60 DAS; while 2 mM SO_4^{2-} application proved inhibitory and lowered the activity by 57.5%, 31.0% and 66.7% at 30 DAS; 96.6%, 85.2% and 173.1% at 60 DAS in Pusa Jai Kisan when compared to their respective control.

In SS2, the increase in CAT, APX and GR activity under NaCl treatment was 17.1%, 47.4% and 33.3% at 30 DAS, 25.8%, 155.8% and 68.6% at 60 DAS, respectively in comparison to their respective control. Application of 1 mM SO_4^{2-} alone resulted in the increase in the activity of above enzymes by 53.9%, 244.6% and 144.4% at 30 DAS, 103.0%, 260.5% and 196.5% at 60 DAS; while 2 mM SO_4^{2-} application proved to be inhibitory and lowered the activity of the enzymes by 11.1%, 10.1% and 43.0% at 30 DAS, 53.0%, 74.4% and 109.3% when compared to their respective control.

In Pusa Jai Kisan, supplementation of 1 mM SO_4^{2-} to NaCl treated plants increased the activity of CAT, APX and GR by 88.4%, 230.5% and 94.4% at 30 DAS and 135.4%, 270.4% and 186.3% at 60 DAS, respectively compared to control. Addition of 2 mM SO_4^{2-} to NaCl-stressed plants further increased the activity of the

enzymes by 113.7%, 260.6% and 133.3% at 30 DAS, 178.1%, 304.6% and 212.8% at 60 DAS compared to control. Similar increase in the activity of the enzymes with the application of 1 or 2 mM SO_4^{2-} was noted in SS2, but the increase was lesser compared to Pusa Jai Kisan.

4.3.6.2 Non enzymatic antioxidants

NaCl treatment significantly decreased the reduced ascorbate content in both the cultivars at both the growth stages, but decrease was higher in SS2 than Pusa Jai Kisan. Supplementation of NaCl treated plants with S lowered the NaCl-induced reduction in reduced ascorbate content in both the cultivars at both growth stages. Application of 2 mM SO_4^{2-} completely alleviated the adverse effect of NaCl on reduced ascorbate content, whereas 1 mM SO_4^{2-} proved less effective (Figures 64-65).

Reduced ascorbate content in Pusa Jai Kisan was decreased by 38.1% and 46.4% at 30 and 60 DAS under 100 mM NaCl treatment compared to control. In SS2, the reduction was 57.7% and 68.1% at 30 DAS and 60 DAS compared to control. Application of 1 mM SO_4^{2-} alone increased the reduced ascorbate content by 7.3% and 10.1% in Pusa Jai Kisan and 3.4% and 6.3% in SS2 at 30 and 60 DAS compared to control. Contrarily, 2 mM SO_4^{2-} decreased the reduced ascorbate content but the reduction was less than 100 mM NaCl alone in both the cultivars.

The supplementation of 100 mM NaCl-treated plants with 1 mM SO_4^{2-} lowered the NaCl-caused decrease in the content of the reduced ascorbate by 12.8% and 28.4% in Pusa Jai Kisan and 26.8% and 36.0% in SS2 at 30 and 60 DAS, respectively compared to control. However, the application of 2 mM SO_4^{2-} to 100 mM NaCl-treated plants ameliorated the NaCl-induced reduction in reduced ascorbate content and improved it by 3.1% and 4.8% in Pusa Jai Kisan and 1.9% and 2.2% in SS2 at 30 and 60 DAS, respectively over the control.

Reduced glutathione content was increased under NaCl treatment in both the cultivars. The increase was higher in Pusa Jai Kisan than SS2. Sulfur either alone or in combination with NaCl further increased the reduced glutathione content of both the cultivars and maximum increase was noted in plants treated with 1 mM SO_4^{2-} and in combination of 2 mM SO_4^{2-} with NaCl at both the growth stages.

Application of 1 mM SO_4^{2-} increased the reduced glutathione content by 80.0% and 135.1% in Pusa Jai Kisan and 68.0% and 126.3% in SS2; while 2 mM SO_4^{2-}

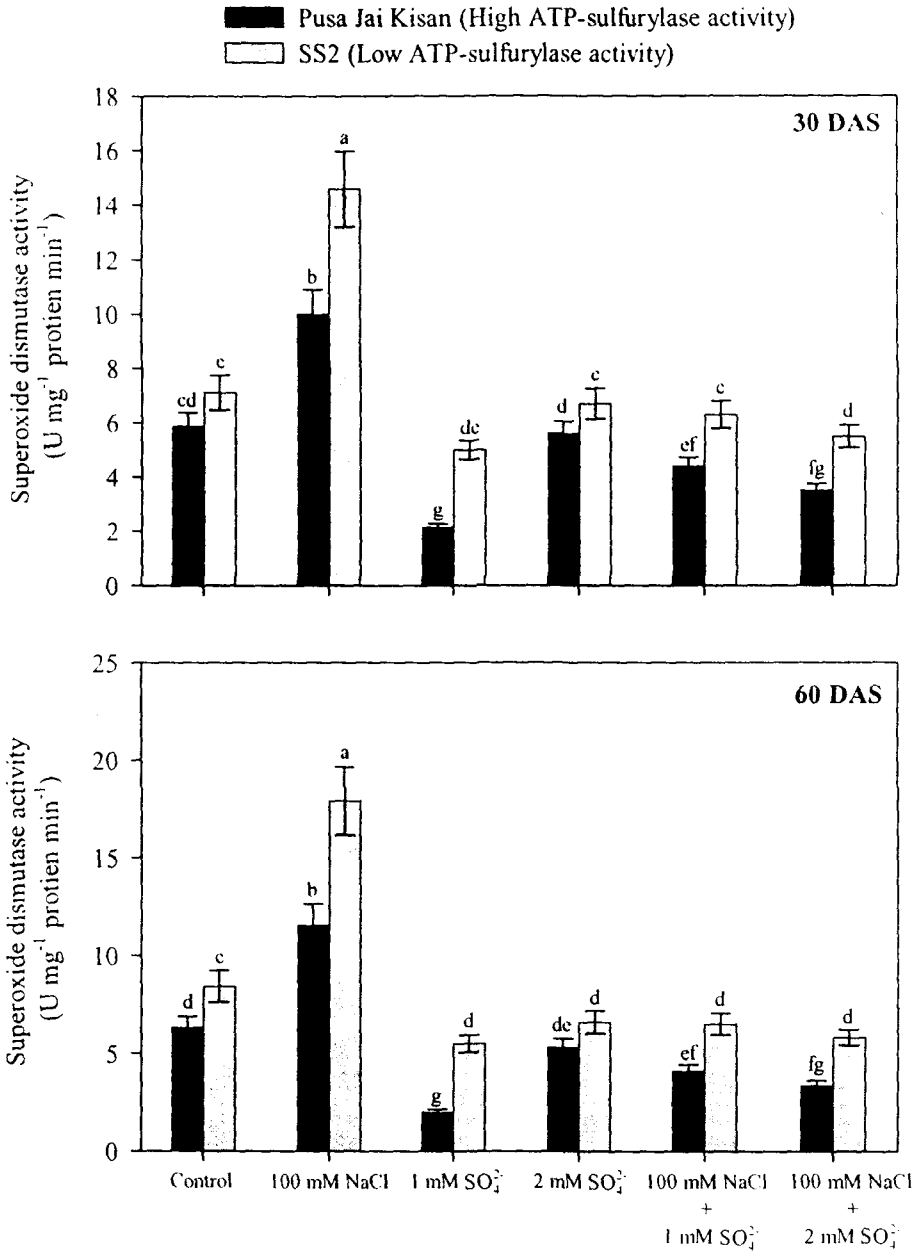

Figure 60. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on superoxide dismutase activity at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

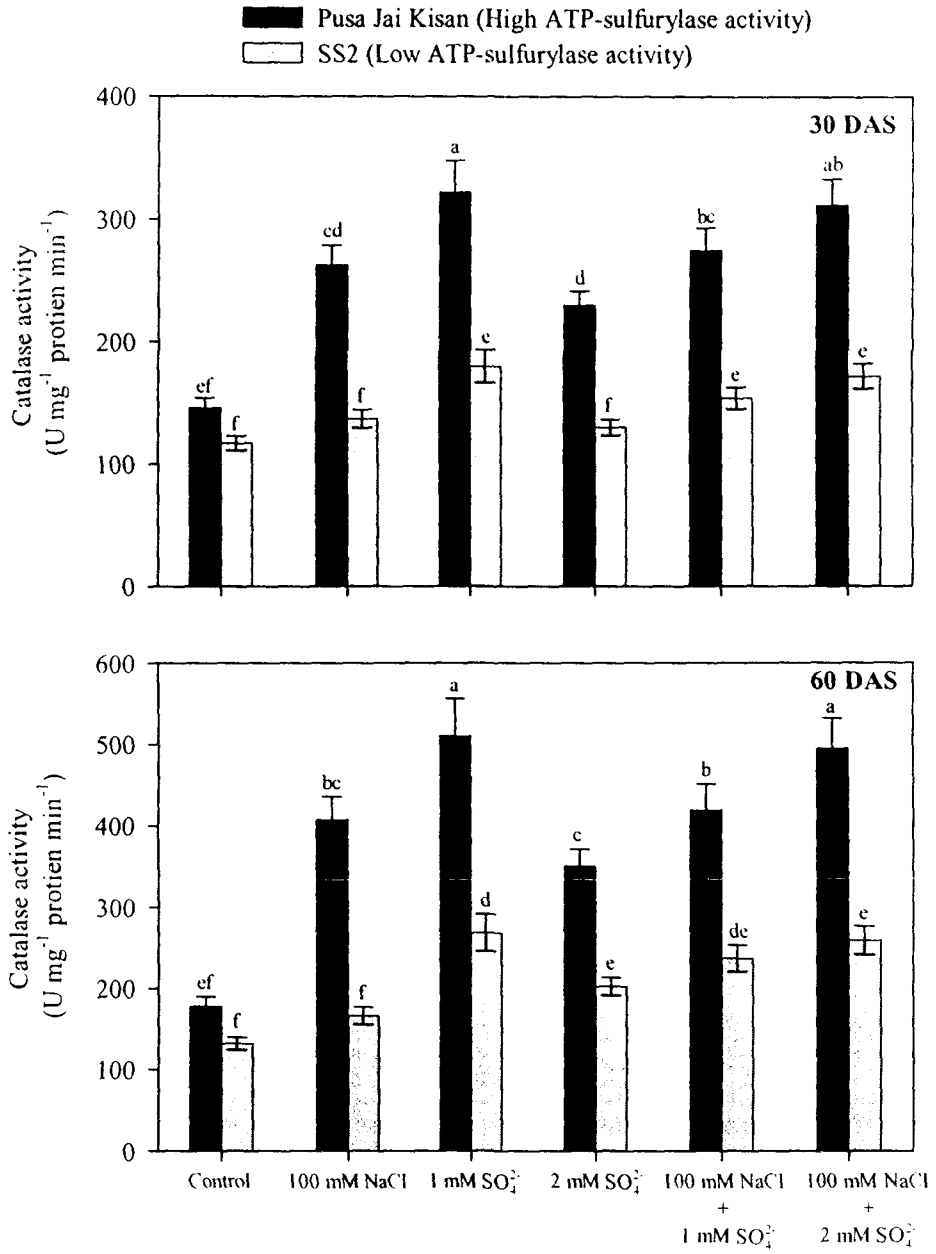

Figure 61. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on catalase activity at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

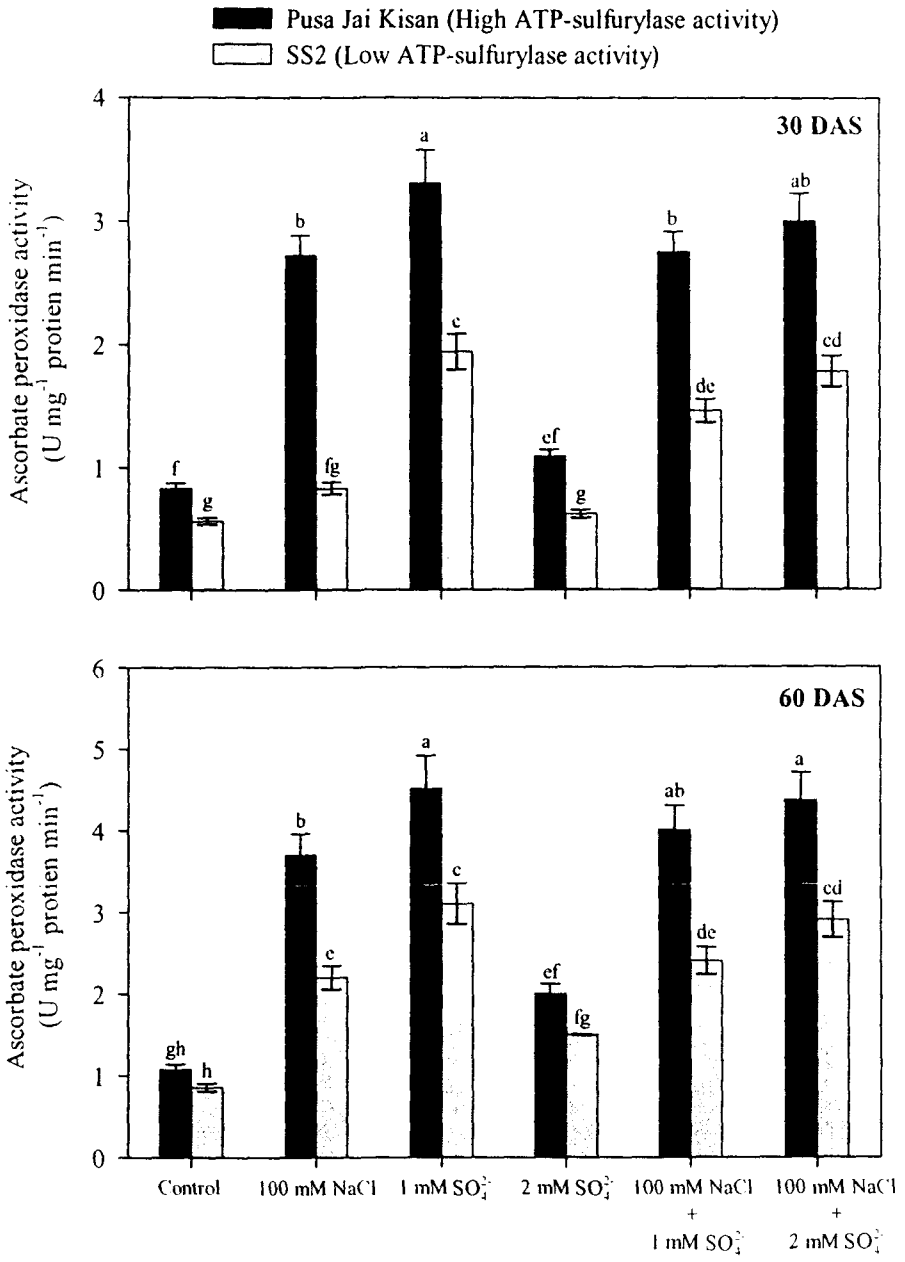

Figure 62. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on ascorbate peroxidise actvity at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

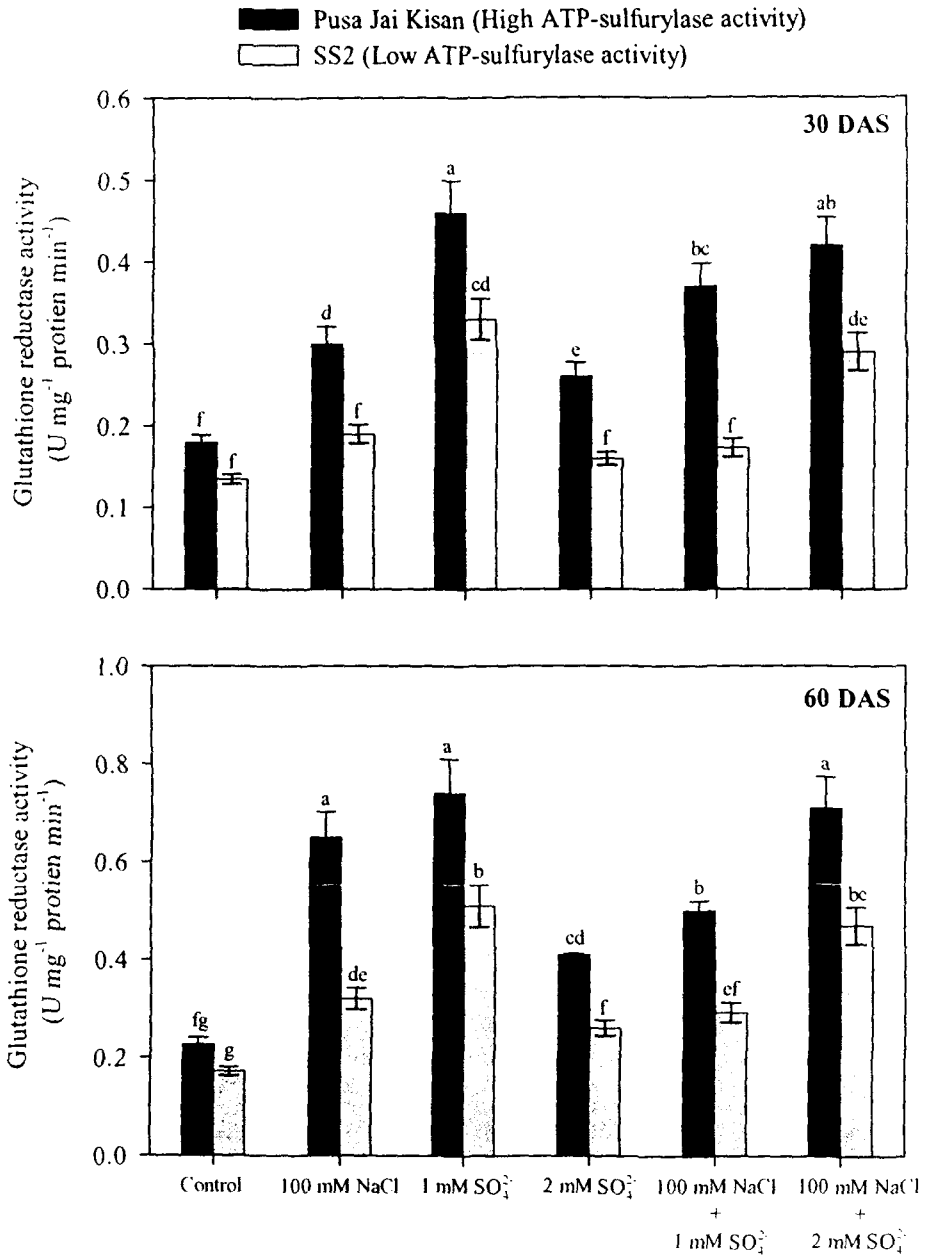

Figure 63. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on glutathione reductase activity at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

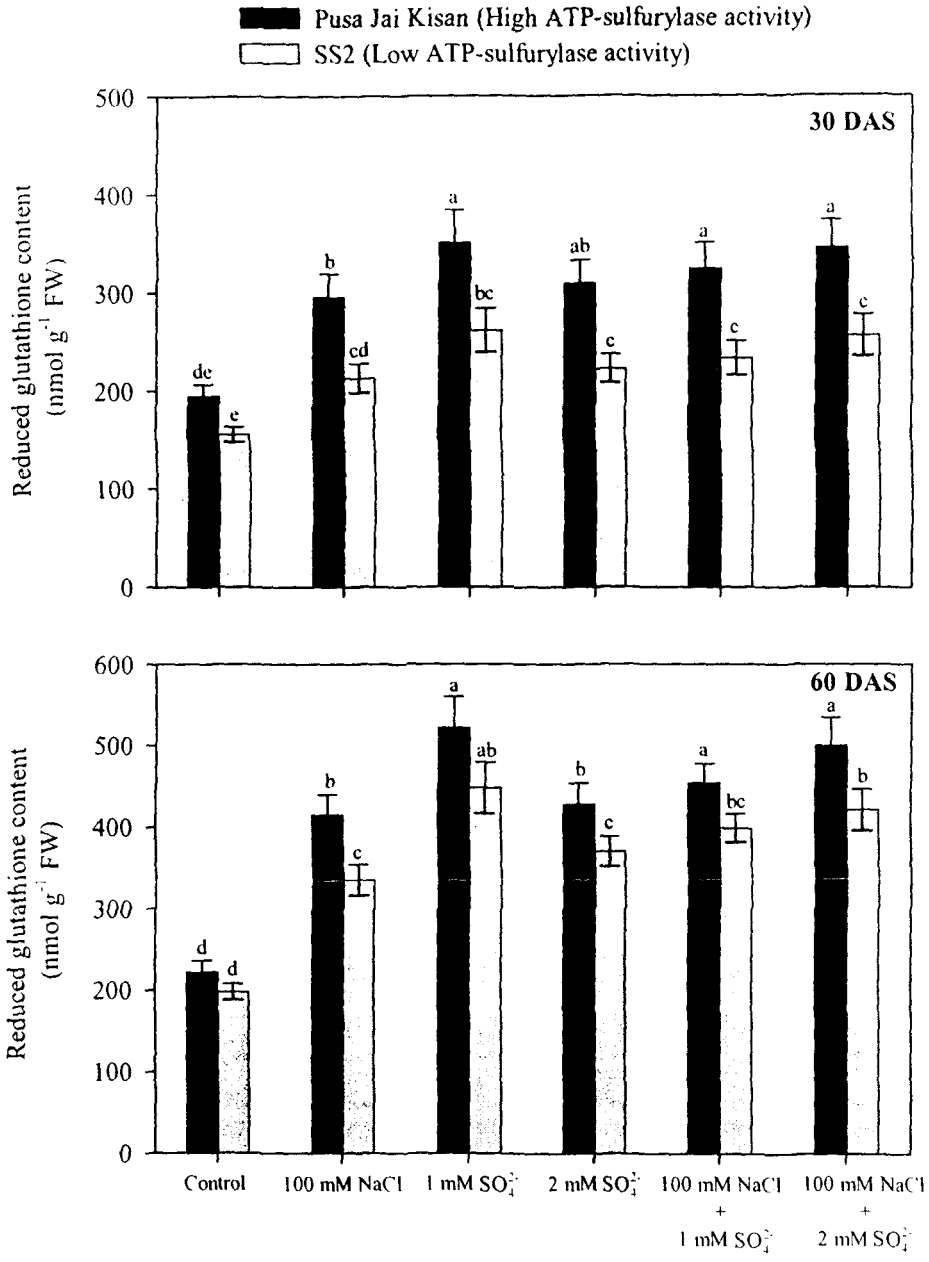

Figure 64. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on reduced glutathione content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

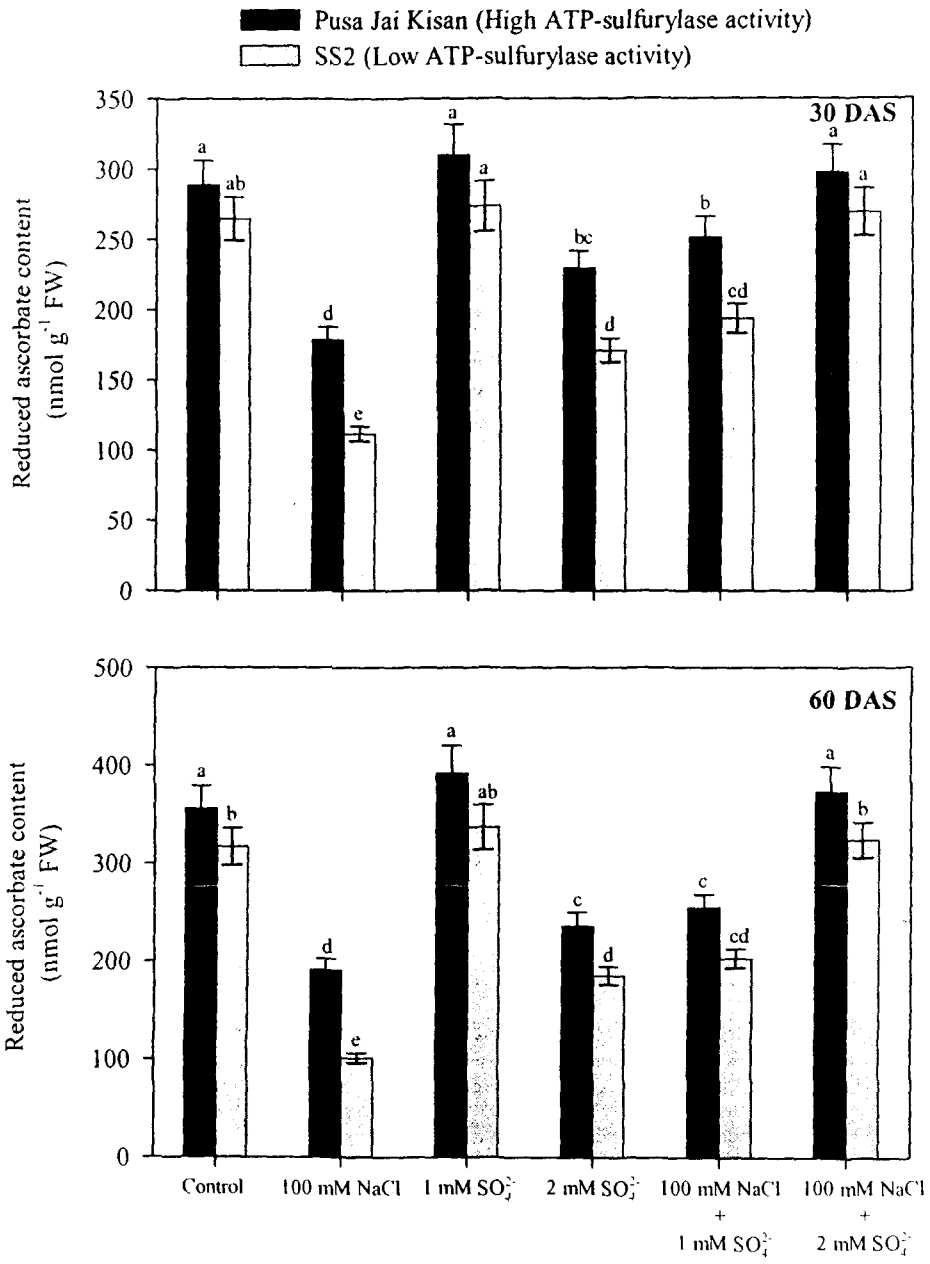

Figure 65. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on reduced ascorbate content at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

application increased it by 59.0% and 92.8% in Pusa Jai Kisan and 43.0% and 86.9% in SS2 at 30 and 60 DAS compared to control.

Supplementation to NaCl-treated plants with S further increased the reduced glutathione content in both the cultivars and maximum significant increase was noted with 2 mM SO_4^{2-}.

4.3.7 Growth characteristics

Plant dry mass and leaf area decreased in both the cultivars treated with 100 mM NaCl both at 30 and 60 DAS. In mustard cultivars grown without NaCl, application of 1 mM SO_4^{2-} exhibited greater values of growth characteristics than 2 mM SO_4^{2-}, while when applied in combination with 100 mM NaCl maximum amelioration of NaCl effects was found with 2 mM SO_4^{2-} in both the cultivars (Figures 66-68). Maximum ameliorative effect was noted in Pusa Jai Kisan.

Maximum significant reduction in plant dry mass and leaf area was noted in SS2 compared to Pusa Jai Kisan at 30 and 60 DAS. In Pusa Jai Kisan the reduction in plant dry mass and leaf area was 46.4% and 38.8% at 30 DAS, 57.6% and 44.5% at 60 DAS, respectively, due to 100 mM NaCl in comparison to control. In SS2, these characteristics were decreased by 57.0% and 47.5% at 30 DAS and 66.7% and 56.1% at 60 DAS in comparison to control.

The application of sulfur alone (without NaCl) was beneficial to the crop growth when applied as 1 mM SO_4^{2-}, however, 2 mM SO_4^{2-} proved inhibitory to both leaf area and plant dry mass in both the cultivars and maximum inhibition was found in SS2.

Application of 1 mM SO_4^{2-} to 0 mM NaCl treated plants increased the plant dry mass and leaf area of Pusa Jai Kisan by 43.5% and 25.0% at 30 DAS, 59.4% and 29.6% at 60 DAS over their respective control. In SS2, plant dry mass and leaf area were increased by 31.6% and 13.0% at 30 DAS, 41.2% and 14.8% at 60 DAS over their respective control. Contrarily, application of 2 mM SO_4^{2-} to non-saline Pusa Jai Kisan decreased the plant dry mass and leaf area by 26.3% and 33.7% at 30 DAS, 35.4% and 38.5% at 60 DAS in comparison to control. In SS2, the plant dry mass and leaf area were decreased by 37.3% and 14.4% at 30 DAS, 47.2% and 54.4% at 60 DAS over their respective control.

Sulfur application, in general proved beneficial in improving growth characteristics under NaCl stress in both the cultivars. In Pusa Jai Kisan, the reductions

in plant dry mass and leaf area caused by 100 mM NaCl was either lowered with 1 mM SO_4^{2-} and/or was completely alleviated by 2 mM SO_4^{2-} application to a greater extent in Pusa Jai Kisan than SS2.

The relative growth rate was decreased by 36.6% and 61.8% in Pusa Jai Kisan and SS2, respectively due to 100 mM NaCl. Application of 2 mM SO_4^{2-} completely reversed the 100 mM NaCl-caused decrease in Pusa Jai Kisan and SS2 (Figure 68).

4.3.8 Yield Characteristics

Salinity stress significantly decreased the yield characteristics (pod number per plant, seed number per plant and seed yield per plant). Application of 1 mM SO_4^{2-} to non-saline plants increased the yield characteristics, whereas 2 mM SO_4^{2-} reduced it but the reduction was lesser than caused by 100 mM NaCl. However, sulfur application (2 mM SO_4^{2-}) to salinized plants completely reversed the NaCl-induced reduction in yield characteristics of both the cultivars.

Pod number per plant, seed number per plant and seed yield per plant of Pusa Jai Kisan was decreased by 36.9%, 23.1% and 35.8%, respectively due to 100 mM NaCl compared to control. Application of 1 mM SO_4^{2-} plus 0 mM NaCl increased the above characteristics by 15.9%, 25.0% and 25.4%, whereas 2 mM SO_4^{2-} reduced these characteristics by 17.2%, 11.5% and 23.9%, respectively over the control. Sulfur supplementation 2mM SO_4^{2-} completely alleviated the NaCl-induced reduction in yield characteristics in both the cultivars (Figure 69).

4.3.9 Summary of Experiment 3

- Application of 2 mM SO_4^{2-} proved most effective in alleviating the effects of 100 mM NaCl stress on growth, photosynthetic, water relations, antioxidant enzymes, oxidative stress and yield characteristics and to a greater extent in Pusa Jai Kisan than SS2.
- Treatment of 100 mM NaCl up-regulated the S assimilation pathway and ATP-sulfurylase activity and S content. Sulfur supplementation to plants treated with 100 mM NaCl further increased the ATP-sulfurylase activity and S content.
- The accumulation of content of TBARS and H_2O_2 due to 100 mM NaCl was lowered by the application of 2 mM SO_4^{2-} in both the cultivars at both the growth stages, whereas 2 mM SO_4^{2-} alone increased the accumulation of TBARS and H_2O_2 but lesser than 100 mM NaCl.

- The supplementation of 100 mM NaCl-treated plants with SO_4^{2-} (1 or 2 mM) further enhanced the NaCl-induced increase in the activity of CAT, APX and GR at both the growth stages in both the cultivars. However, Pusa Jai Kisan exhibited maximum increase in the activity of above mentioned enzymes due to application of S (1 or 2 mM SO_4^{2-}) to NaCl-treated plants.
- Taking above results together, it is clear that compared to SS2, Pusa Jai Kisan showed maximum response to the S supplementation (1 or 2 mM SO_4^{2-}).
- Under non-saline condition application of 1 mM SO_4^{2-} proved more beneficial to the cultivars than 2 mM SO_4^{2-}, but application of 2 mM SO_4^{2-} was more beneficial under salinity stress.

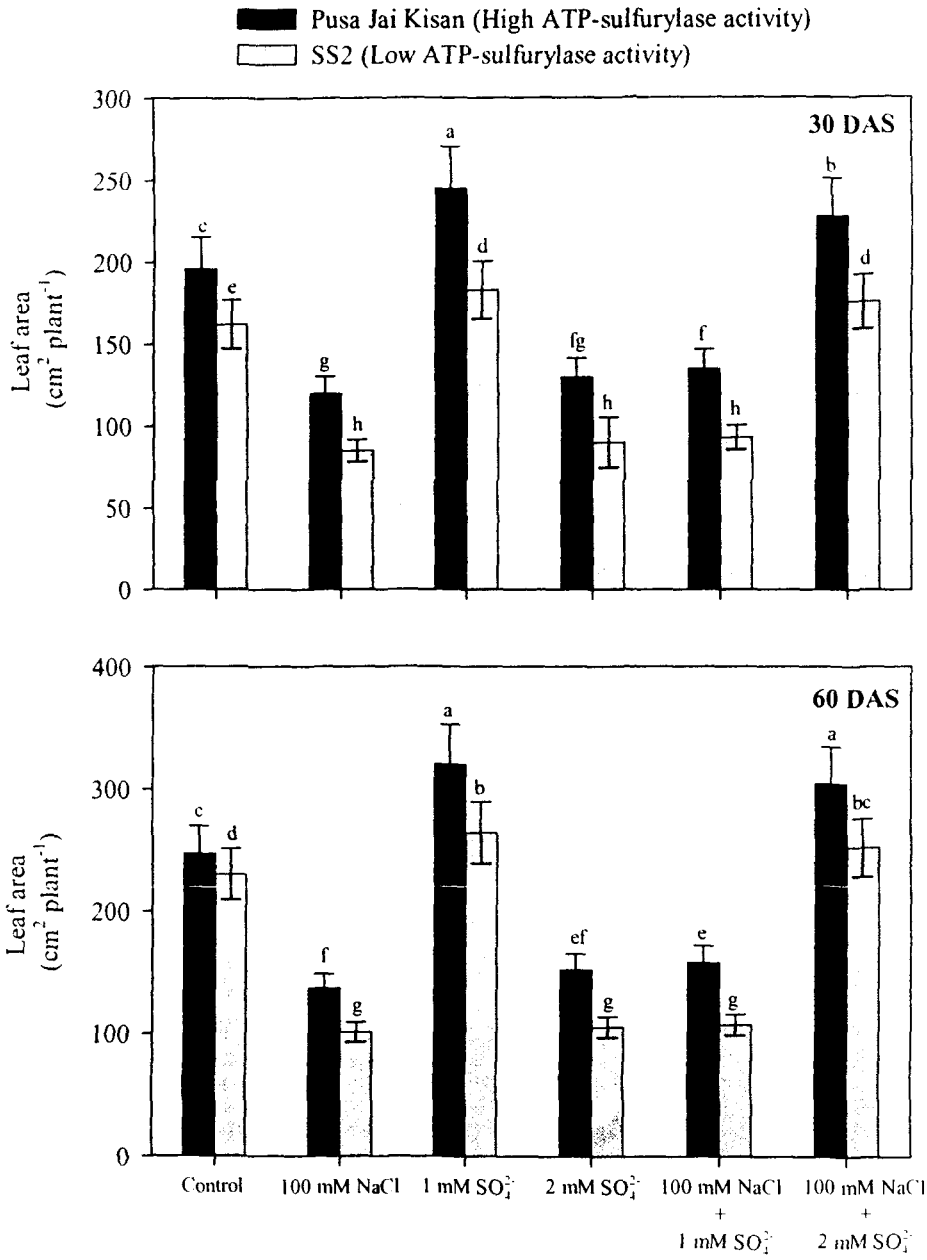

Figure 66. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on leaf area at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

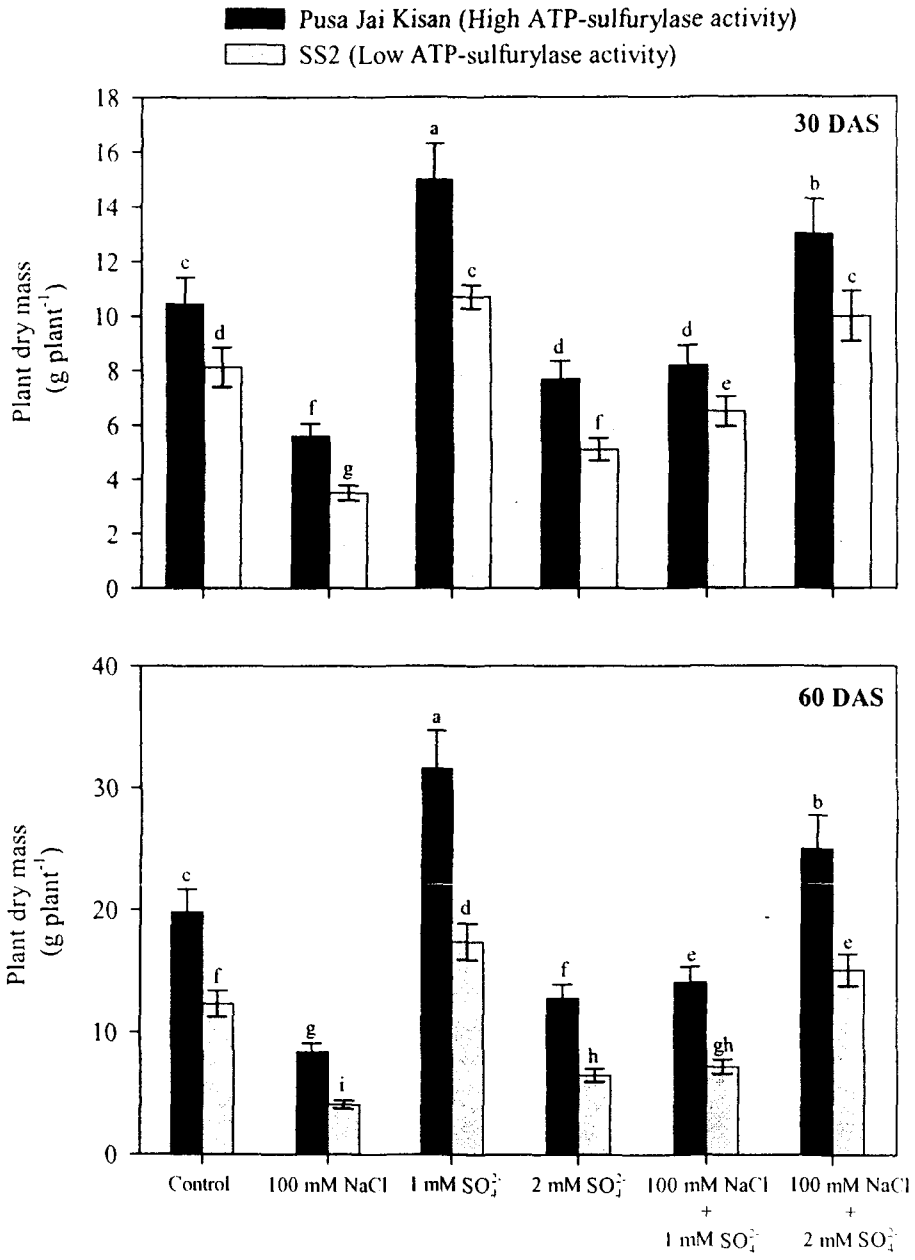

Figure 67. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on plant dry mass at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

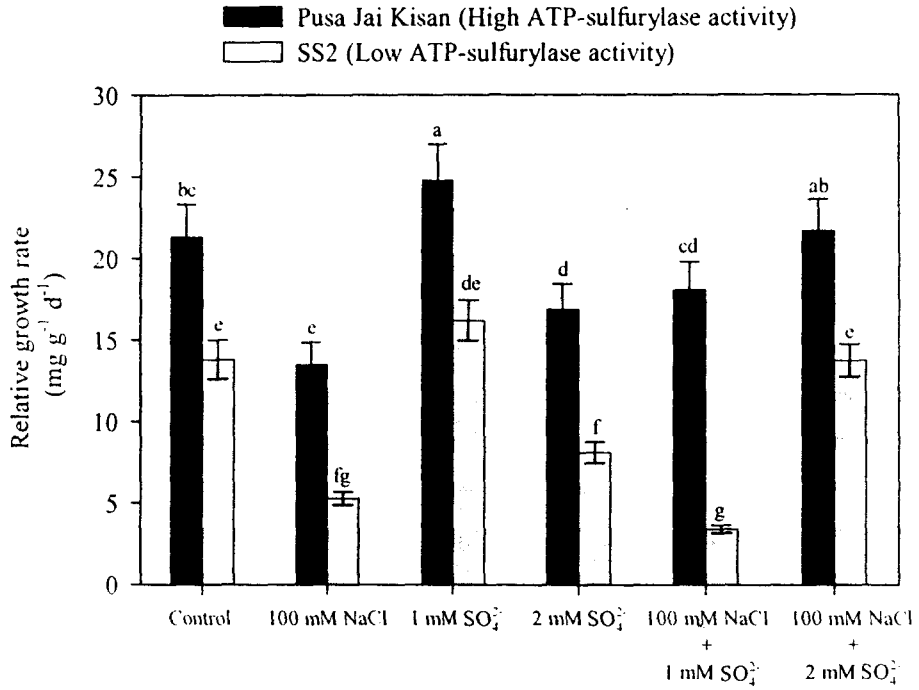

Figure 68. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on relative growth rate at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

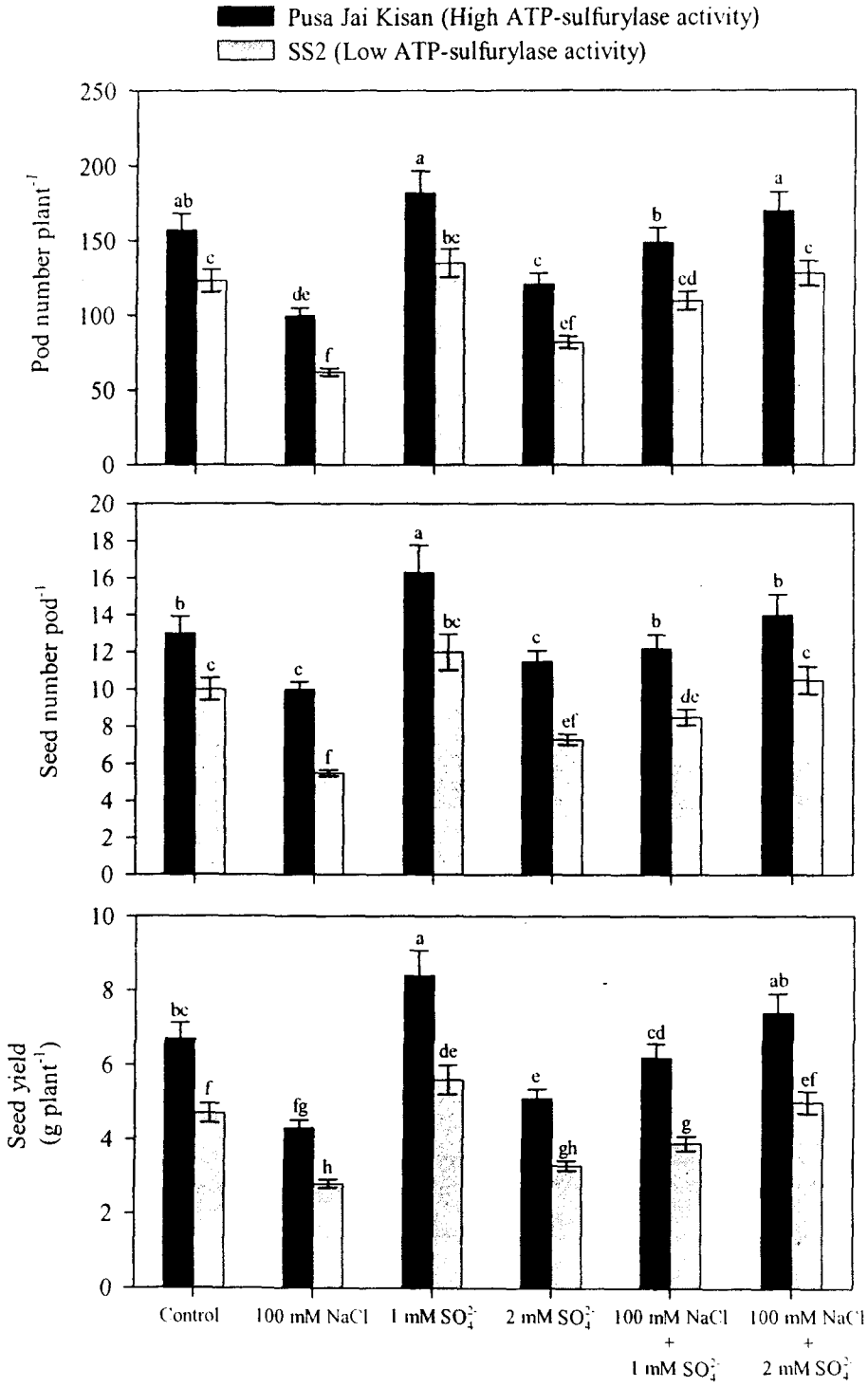

Figure 69. Response of two mustard (*Brassica juncea* L.) cultivars to NaCl and sufur (SO_4^{2-}) applied alone or in combination on pod number per plant, seed number per plant and seed yield at 30 and 60 DAS. Data are Mean ± S.E. (n = 3). Bars showing the same letter are not significantly different at $p<0.05$ as determined by LSD test.

Discussion

Contents

DISCUSSION

5.1 Introduction

Environment is an integral component of the biosphere. Any change in its components is bound to have a direct impact on the biota of ecological system and their adaptive mechanisms. In India, reduction in crop productivity by abiotic stresses results in the loss of hundreds of millions of rupees every year (Mahajan and Tuteja 2005). Among abiotic stresses, salinity stress is the most severe environmental stress, impairing crop production at least 20% of irrigated land worldwide. In addition, the increased salinity of arable land is expected to have global effects and may result in the loss of up to 50% fertile land by the middle of the twenty first century (Flowers and Yeo 1995; Zhu 2001; Mahajan and Tuteja 2005; Manchanda and Garg 2008). This is due to the fact that vast area of the available land on the globe is expected to be affected by salinity-induced adverse effects in plants, which will have (i) ionic effect (ii) osmotic effect (iii) nutrient imbalance (iv) hormonal imbalance and (v) production of ROS (Ashraf and Foolad 2007; Ashraf 2009).

Briefly, it may be reiterated that salinity is the presence of excessive concentrations of soluble salts in soil solution. The salts are chlorides and/or sulphates of calcium, magnesium, sodium and potassium. Among these chloride is usually predominant (Brady 2002). As a consequence of considerably higher evaporation than total precipitation in arid and semi-arid regions, upward water movement results in the accumulation of salts in the root zone. With the increase in salinity level plants extract water less easily from soil, aggravating water stress conditions. High soil salinity also causes nutrient imbalance resulting in the accumulation of toxic elements and reducing water infiltration if the level of sodium is high.

The effects of salts excess in the root environment on the physiology of plants have been studied for over 100 years, and two major components of salt stress have been identified: the osmotic and the ionic. After stressor recognition by the root cells, there is transduction of the signal and a series of changes occur in the metabolism. These changes in the metabolism result in the production of transport proteins involved in ion exclusion and compartmentalization (Zhu 2001; Taiz and Zeiger 2002) and of organic solutes, which are involved in osmotic and ionic homeostasis in the

osmoprotection and cell detoxification (Bray *et al.* 2000). The salinity-induced changes in metabolism have also shown to influence hormonal balance (Prisco and O'Leary 1972; Taiz and Zeiger 2002) causing increase in the production of ROS, which act as a secondary messenger (Bray *et al.* 2000).

The generation of ROS is of common occurrence among the effects of biotic and abiotic stresses. Since ROS are highly reactive in nature, these have been shown to cause oxidative damages to lipids, proteins, nucleic acids and affect cell membrane properties. Therefore, plant tolerance to salinity and to other abiotic stress depends on the development of efficient mechanisms that can tightly regulate the ROS generation and/or scavenging of ROS generated in excess.

The success of the sustainable crop production relies mainly on the use of techniques and/or methods which can alleviate adverse effects of various stress factors in plants and maintain crop productivity as well. For sustainable agriculture it has to be taken in mind that how plants responds to environmental stress, and stress response is dependent on several factors including sensitivity of cultivars to the stress (Figure 70). Several strategies have been suggested to counteract the NaCl-induced growth inhibition by reducing the accumulation of deleterious ions Na^+ and Cl^- in plant parts (Sairam and Tyagi 2004; de Abreu *et al.* 2008; Manchanda and Garg 2008; Moud and Maghsoudi 2008). Increasing evidence suggests that mineral nutrient status of plants plays a critical role in increasing plant resistance to environmental stresses (Marschner 1995; Hassan *et al.* 2005a, b; 2008a, b; Vassilev *et al.* 2005; Anjum *et al.* 2008a, b; Khan *et al.* 2008).

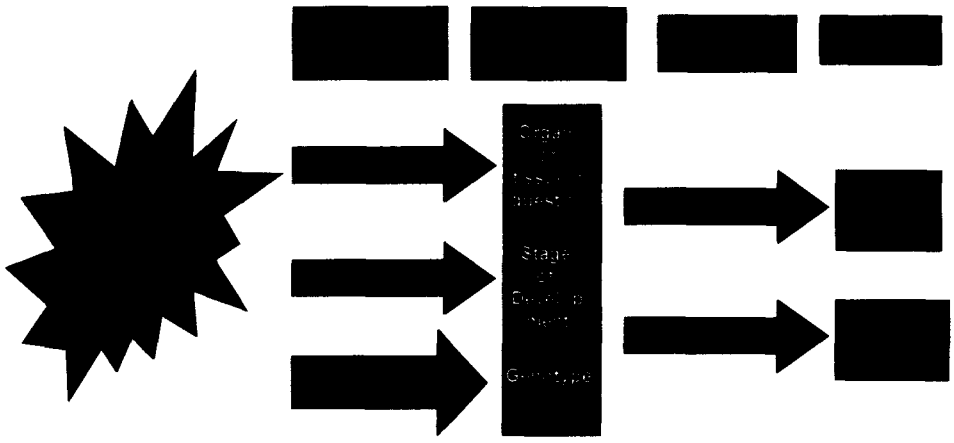

Figure 70. Various factors influencing response of plants to stress.

Sulfur is an essential macronutrient that plays a vital role in the regulation of plant growth and development (Marschner 1995, Ernst 1998; Anjum *et al.* 2008a; Nazar *et al.* 2008; Ratti and Giordano 2008; Khan *et al.* 2009a) and required in higher quantity by mustard (Zhao *et al.* 1993; Lakkineni and Abrol 1994; McGrath and Zhao 1996). It is a precursor of cysteine and methionine, amino acids involved in the synthesis of compounds containing reduced S (Marschner 1995; Scherer 2001). A sufficient S supply improves photosynthesis and growth (Ahmad *et al.* 2005; Khan *et al.* 2005) through regulating N assimilation (Reuveny *et al.* 1980; Kopriva *et al.* 2002; Scherer 2008). A large accumulation of N maintains high chlorophyll content and high activity of enzymes of the Calvin cycle (Lawlor *et al.* 1989), and enhances growth (Schnug *et al.* 1993; Khan *et al.* 2005). The assimilatory pathways of S and N have been considered functionally convergent and well coordinated as the availability of one element regulates the other (Reuveny *et al.* 1980; Schnug *et al.* 1993). The availability of S regulates the activity of nitrate reductase and the accumulation of N (Pal *et al.* 1976). Sulfur is a structural constituent of several coenzymes and prosthetic groups, such as ferredoxin, which are also important for N assimilation. The first step of S assimilation in plants is the activation of S by the enzyme ATP-sulfurylase, and in a cascade of enzymatic reactions inorganic S is converted to non-protein thiol GSH (Tausz *et al.* 2004; Szalai *et al.* 2009). Glutathione has been shown to take part in the removal of excess H_2O_2 (Noctor and Foyer 1998) and lipid peroxidases. Thus keeping ROS under control (Rausch *et al.* 2007; Srivalli and Khanna-Chopra 2008) and protecting plants from oxidative damage.

Keeping in view the importance of S in plant development under normal and abiotic stress conditions, the present work was taken up with the following objectives:

- To screen and select the S-efficient and S-inefficient mustard cultivars by studying the activity of ATP-sulfurylase enzyme and S accumulation
- To study the physiological response of high ATP-sulfurylase activity and low ATP-sulfurylase activity cultivars of mustard to salinity stress
- To study the influence of sulfur in the alleviation of salinity-induced adverse effects in mustard cultivars with high and low ATP-sulfurylase activity.

5.2 Screening of mustard cultivars differing in ATP-sulfurylase activity and S accumulation capacity/ S-efficiency

Experiment I was conducted to select the S-efficient and S-inefficient mustard cultivars by studying the activity of ATP-sulfurylase enzyme and S accumulation, photosynthesis and growth of Pusa Jai Kisan, Alankar, Varuna and SS2 cultivars of mustard.

All the cultivars differed in ATP-sulfurylase activity, content of S and N, photosynthetic rate, growth characteristics and STI, being maximum in Pusa Jai Kisan and minimum in SS2. Further, a strong positive correlation ($P<0.01$) was found between ATP-sulfurylase activity and photosynthesis and shoot dry mass (Figure 3).

ATP-sulfurylase catalyzes the first step of S assimilation pathway and therefore may act as a point of regulation in S uptake and its subsequent reduction (Hawkesford and Wray 2000). ATP-sulfurylase activity in plants was first demonstrated by Asahi (1964) and found in multiple species, including maize, spinach, *Arabidopsis thaliana*, soybean, potato and tobacco (Leustek *et al.* 2000). The activity of the enzyme is highest in the expanding leaves and declines rapidly after leaf maturation (Cacco *et al.* 1977). Genes encoding chloroplastic, mitochondrial, and cytosolic ATP-sulfurylase isoforms have been cloned from *Arabidopsis, Brassica juncea* and *Solanum tuberosum* (Klonus *et al.* 1994; Leustek *et al.* 1994; Logan *et al.* 1996; Heiss *et al.* 1999; Hatzfeld *et al.* 2000).

In the present study, the cultivar Pusa Jai Kisan exhibited maximum ATP-sulfurylase activity and also photosynthetic rate and plant growth. A higher ATP-sulfurylase activity and sulfate-transport index in Pusa Jai Kisan indicates its higher sulfate accumulation capacity. An increased accumulation of sulfate helped in the greater N content. As ATP-sulfurylase activity was greatest in Pusa Jai Kisan, the sulfate accumulated was assimilated efficiently into reduced S resulting in leaf sulfate content lesser than in Alankar. This resulted in higher leaf sulfate content in Alankar than Pusa Jai Kisan. A higher accumulation of sulfate resulted in higher N content and photosynthetic rate in Pusa Jai Kisan as N is necessary for high contents of chlorophyll and ribulose-1,5-bisphosphate carboxylase protein (Lawlor *et al.* 1989). Ahmad and Abdin (2000) and Ahmad *et al.* (2005b) have also found increased photosynthetic rate

with adequate S supply, and inadequacy of S caused the accumulation of non-protein N in the vegetative tissue at the expense of protein N. The higher activity of ATP-sulfurylase and N accumulation in Pusa Jai Kisan also resulted in the development of larger leaf area. Larger leaf area in Pusa Jai Kisan intercepted more photons and efficiently utilized solar radiation resulting in higher photosynthetic rate. Photosynthetic rate integrated over time and leaf area resulted in the increased dry mass accumulation. This suggests that the cultivar Pusa Jai Kisan possesses greater S accumulation and assimilation capacity which was possibly associated with the higher sulfate transporter system. The difference in sulfate uptake and S transporter system in mustard genotypes have been shown by Ahmad *et al.* (2005a). However, an association of ATP-sulfurylase activity with photosynthesis and shoot dry mass has not been reported. A strong positive correlation observed between the activity of ATP-sulfurylase and photosynthetic rate and shoot dry mass in all four cultivars (Figure 3) suggests their concomitant relation. Hence, the activity of ATP-sulfurylase may be used as a physiological trait for augmenting photosynthesis and shoot dry mass accumulation in mustard.

On the basis of overall performance of the *Brassica juncea* cultivars tested in Experiment 1, Pusa Jai Kisan emerged as high ATP-sulfurylase activity cultivar and SS2 as low ATP-sulfurylase activity cultivar.

5.3 Study on the effect of salinity stress and its alleviation by sulfur in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (high ATP-sulfurylase activity) cultivars of mustard

The physiological effects of salinity stress on growth and metabolism of plants have been detailed out in the chapter 'Review of Literature'. In the following pages efforts have been made to discuss the role of sulfur in the alleviation of salinity-induced adverse effects. Experiment 2 was conducted to assess the effects of salinity stress in Pusa Jai Kisan and SS2 and Experiment 3 was conducted to study how sulfur ameliorates NaCl-induced toxicity. In Experiment 2, both the cultivars of *Brassica juncea* were treated with 0, 50, 100 mM NaCl. Plants subjected to 50 and 100 mM NaCl exhibited reduced growth, photosynthetic, water and osmotic potential, contents of nutrient and yield characteristics in comparison to control. The effect of 100 mM

NaCl was found more pronounced which decreased the growth and photosynthetic characteristics maximally. Therefore, Experiment 3 was aimed at analyzing the amelioration of 100 mM NaCl-induced effects with sulfur nutrition (1 and 2 mM SO_4^{2-}) by studying sulfur assimilation, photosynthetic traits, water relations, contents of nutrients and ions, oxidative stress, various components of antioxidant defense system, growth and yield in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (low ATP-sulfurylase activity).

The following sections discuss the major results of Experiments 2 and 3 in the light of available recent literature and also possible explanations have been made.

5.3.1 ATP-sulfurylase activity and S accumulation

In the present study, the salinity stress increased the ATP-sulfurylase activity and S accumulation in both the cultivars at both the growth stages (Figure 4). Pusa Jai Kisan exhibited higher ATP-sulfurylase activity than SS2 at both NaCl levels. However, the effect of 50 and 100 mM NaCl on ATP-sulfurylase activity did not differ significantly in both the cultivars. Salinity stress-induced increase in ATP-sulfurylase activity and S content could be a possible consequence of an increased requirement of glutathione under salinity stress. In this context, sulfur plays a pivotal role because sulfate transporters can mediate the entry of sulfate-analogues into cells (Maruyama-Nakashita *et al.* 2007). Sulfur assimilation in plants starts with the activation of sulfur by ATP-sulfurylase, and in a cascade of enzymatic reactions inorganic sulfur is converted to non-protein thiol glutathione (Tausz *et al.* 2004). GSH plays an important role in response to various biotic and abiotic stresses as it is a major thiol-disulfide redox buffer in plant cells (May *et al.* 1998a, b; Schafer and Buettner 2001) and is dependent on the availability of cysteine, which in turn is dependent on sulfate assimilation. Earlier, we have reported that higher expression of ATP- sulfurylase activity is necessary for the maintenance of optimal GSH levels required for the proper functioning of ascorbate-glutathione cycle in mustard (Khan *et al.* 2009a).

Sulfur also has other physiological roles in the biosynthesis of lipids and proteins and is required for the synthesis of various other compounds, sulfolipids and secondary sulfur compounds (alliins, glucosinolates, phytochelatins), which play an important role in the physiology of plants and in the protection and adaptation of plants

against stress. The uptake and assimilation of sulfur and nitrogen are strongly interrelated and dependent upon each other (Brunold 1993). Besides, proteins contain both sulfur and non-sulfur amino acids and for this reason the availability of nitrogen and sulfur interacts with the utilization of nitrogen and sulfur for proteins and plant growth. The organic N/S ratio of plants and seeds reflects the sulfur status of the plant. At a sufficient sulfur supply the organic N/S ratio is generally around 20 (Dijkshoorn and Van Wijk 1967; Brunold 1993; De Kok *et al.* 2000). It has been shown that sulfur deficiency results in the loss of plant fitness, plant's resistance to environmental stress and pests and in decreased food quality and safety (De Kok *et al.* 2002). The exact mechanism of the effect of sulfur supplementation on salt stress tolerance is still not known so far.

The application of S (2 mM SO_4^{2-}) in Experiment 3 further increased the ATP-sulfurylase activity and S content of NaCl treated plants but the extent of increase was greater in the Pusa Jai Kisan than the SS2. Non-salinized plants treated with 1mM SO_4^{2-} maintain high ATP-sulfurylase activity and S content whereas 2 mM SO_4^{2-} proved less effective. Our results suggest that maximum activity of ATP-sulfurylase can only be achieved with a suitable S supply (1 mM SO_4^{2-}) to the plants under normal condition, and under salinity stress there is a higher requirement of S in plants.

The involvement of S in salinity tolerance has not been reported. However, in case of Cd tolerance the importance of S has been reported in *Arabidopsis thaliana* (Dominguez-Solis *et al.* 2001; Harada *et al.* 2002), *Brassica juncea* (Zhu *et al.* 1999a, b), *Nicotiana tabacum* (Harada *et al.* 2001), *Triticum aestivum* (Khan *et al.* 2007a) and *B. campestris* (Anjana *et al.* 2006; Anjum *et al.* 2008a). A good part of S incorporated into organic molecules in plants is located in thiol (-SH) groups in proteins (cysteine-residues) or non-protein thiols (glutathione) (Noji and Saito 2003; Tausz *et al.* 2003; De Kok *et al.* 2005; Anjum *et al.* 2008b). Chen and Huerta (1997) showed that S is a critical nutritional factor for reduction of Cd toxicity. In fact, different abiotic stresses are often interconnected and cause similar cellular damage. The plant responses to these stresses are complex and multifold. To prevent damage, the antioxidant response can be crucial in plants. Although glutathione has multiple roles in plant cells, its function as an antioxidant is an important one. A glutathione deficient mutant of

Rhizobium has been shown to be sensitive to osmotic and oxidative stresses and even weak organic acids (Riccillo *et al.* 2000).

5.3.2 Photosynthetic characteristics

Both the NaCl levels used in the study significantly reduced the photosynthetic characteristics in both the cultivars. However, the extent of the decrease in these characteristics was more conspicuous in SS2 at both the sampling times compared to Pusa Jai Kisan (Figure 6). Photosynthetic characteristics decreased maximally with 100 mM NaCl in both the cultivars in comparison to control. The response of photosynthetic traits to various stress factors are good indicators of physiological stress levels in all plants. In general, reduction in photosynthesis and plant dry mass with increased salinity could be attributed to the difference in the efficiency of root system in limiting the transport of ions to shoots (Munns *et al.* 2006) and to induced water deficit in plant parts (Abed Alrahman *et al.* 2005). The inhibition of photosynthesis under salinity stress may be attributed to water deficit-induced stomatal closure (Steduto *et al.* 2000; Meloni *et al.* 2003) in addition to several other biochemical and photochemical processes (Chen *et al.* 1999; Sultana *et al.* 1999). The decrease in photosynthesis may be considered as one of the most important factors responsible for reduced plant growth and productivity under high salinity conditions (Ball *et al.* 1987) as salinity has an influence on sink strength through changes in carbohydrate partitioning and accumulation (Paul and Foyer 2001). Salinity-caused reductions in photosynthetic pigments and stomatal conductance that directly and/or indirectly impair photosynthesis have been shown (Flexas *et al.* 2004; 2007). A significant reduction in photosynthesis, stomatal conductance and chlorophyll pigments was observed under high salinity stress in *Pueraria lobata* (Al-Hamdani 2004). Leaf photosynthetic capacity depends on physiological characteristics such as chlorophyll contents, Rubisco activity, and photosystem efficiency (Chen *et al.* 1999). Reduction in the chlorophyll content of the plant is accompanied by a lower efficiency of PS II and senescence. Another possible factor contributing to decreased photosynthesis is the inhibitory effect of salt stress on the efficiency of translocation and assimilation of photosynthetic products (Ma *et al.* 1997; Chen *et al.* 1999; Demetriou *et al.* 2007). In addition, salinity decreases whole plant photosynthesis by restricting leaf area expansion (Netondo *et al.*

2004). A significant reduction in CO_2 assimilation rate and stomatal conductance by salt treatment is registered in horse gram (*Macrotyloma uniflorum*). Salinity stress has been shown to inhibit photosynthetic electron transport and the activity of the Calvin cycle (Reddy *et al.* 1992). In the present investigation, NaCl significantly decreased the photosynthetic characteristics in SS2 than Pusa Jai Kisan suggesting that the inhibition of photosynthesis under NaCl stress may be attributed to stomatal closure due to water deficit.

Application of 1 mM SO_4^{2-} alone significantly increased the photosynthetic characteristics in both the cultivars in comparison to 2 mM SO_4^{2-}. However, supplementation of 2 mM SO_4^{2-} completely ameliorated the NaCl-induced toxicity and improved the photosynthetic characteristics, but 1 mM SO_4^{2-} proved less effective in the alleviation of salinity stress. Resurreccion *et al.* (2001) reported that S application increased the chlorophyll and Rubisco content which led to increased photosynthesis of *Oryza sativa*. Khan *et al.* (2005) have also reported that S application increased the relative growth rate, plant growth rate, net assimilation rate and carbon dioxide exchange rate of *Brassica juncea* plants. At the cellular level, S starvation reduces the mesophyll cell number per cm^2 and the chlorophyll content per chloroplast.

Transpiration rate increased with the increase in NaCl level and was found maximum with 100 mM NaCl treatment. SS2 exhibited higher transpiration rate in comparison to Pusa Jai Kisan under salinity stress. The lower transpiration rate of Pusa Jai Kisan was due to its ability to minimize water loss under low water potential conditions of saline soil. This maintained higher photosynthetic rate in Pusa Jai Kisan than SS2 under salinity stress. Koyro (2006) and Geissler *et al.* (2009) have also shown plants exhibiting low transpiration rate together with high photosynthesis. Plants develop strategies for tightly controlled phenomenon of salt uptake, accumulation and transport within the plant to avoid the problem of ion stress. Under such conditions plants cells tend to readjust their osmotic potential to prevent water loss. This is achieved either by uptake of inorganic ions from the external medium, or by *de novo* synthesis of compatible solutes.

In the current study, the application of 1 mM SO_4^{2-} alone reduced the transpiration rate in non-salinized plants, whereas 2 mM SO_4^{2-} proved less effective.

Contrarily, under salinity stress application of 2 mM SO_4^{2-} reduced the effects of NaCl on transpiration rate. It implies that the excess dose of S (2 mM SO_4^{2}) alone which proved to be toxic to plants grown without NaCl proved beneficial when applied under salinity stress. It may be explained as that excess of sulfur is utilized by the plants to maintain salt transport and ion homeostasis, which is maintained by the increased stomatal resistance. This results in reduced transpiration, salt transport and accumulation (Everard *et al.* 1994).

5.3.3 Water relations

In our study, leaf water potential and osmotic potential decreased in both cultivars but significant decrease in these traits was noted in SS2 compared to Pusa Jai Kisan under 100 mM NaCl. Reduction in leaf water potential with NaCl treatment has also been previously also been observed by Yazici *et al.* (2007) and da Silva *et al.* (2008). The higher decrease in water potential in SS2 than Pusa Jai Kisan resulted in closure of stomata and reduced the availability of CO_2. Subsequently, greater alteration in the efficiency of PSII and electron transport chain as evident by significant fall in the ratio of Fv/Fm of the dark adapted SS2 leaves contributed to higher reduction in photosynthesis than Pusa Jai Kisan under salinity stress. Salinity stress has been found to decrease the PSII activity (Lu and Vonshak 2002) and inhibit the quantum yield of PSII electron transport (Xia *et al.* 2004). Reductions in leaf water potential and osmotic potential have been shown to reduce stomatal conductance and eventually inhibit photosynthetic metabolism (Baker and Rosenqvist 2004; Zribi *et al.* 2008). Moreover, stomatal closure may also lead to increased susceptibility to photo-damage (Powles 1984). It is known that salt stress reduces hydraulic conductance in roots, resulting in decrease of water flow from root to shoot. Thus, under such conditions there is an alteration in water relations even in osmotically adjusted plants (O'Leary 1994; da Silva *et al.* 2008).

The effect of 1 mM SO_4^{2-} on leaf water potential and osmotic potential of Pusa Jai Kisan and SS2 cultivar increased under non-saline condition, whereas 2 mM SO_4^{2-} proved most effective in increasing water and osmotic potential of NaCl treated plants. Hence, the improved S nutrition allowed a more adequate plant defense to NaCl toxicity and also prevented S deficiency in plants.

5.3.4 Nutrients and Ions

The internal transport of plant nutrients largely depends on the synthesis, utilization and translocation of photosynthates. Any perturbation in the system may severely affect both the supply and the demand of nutrients for crop plants (Alam 2001). In the present study, the content of N, P, K and Ca decreased significantly with NaCl treatment in both the cultivars, but the decrease was found maximum in SS2. In both the cultivars, decreased N, P, K and Ca content due to 100 mM NaCl was completely ameliorated with the supplementation of 2 mM SO_4^{2-}, whereas 1mM SO_4^{2-} proved less effective. However, under non-saline condition application of 1 mM SO_4^{2-} significantly increased the nutrients content in both the cultivars, whereas 2 mM SO_4^{2-} on contrast reduced the nutrients content. Hence, proper S supply has positive effects in overcoming the adverse effects caused by NaCl stress.

The S requirement of crops depends on several factors. Of which the balance between S and other nutrient element is important in view of a possible synergistic or antagonistic effect. Clarkson *et al.* (1989) observed a marked depression in the ability of cereal plants to take up nitrate and ammonium when plants were starved with S.

Potassium content in leaf and root tissues was much lower in salinity-stressed plants under 100 mM NaCl and induced a significant decrease in K uptake. Raptan *et al.* (2001) and Tuna *et al.* (2008) have also reported that salinity decreased K availability. Epstein (1966) showed antagonistic relationship between K and Na uptake. Therefore, the role of K and S in augmenting yield and improving the quality of crops is well known (Singh and Rathore 1994; Prasad *et al.* 1996; Razmjoo and Henderlong 1997; Umar *et al.* 1997).

The interaction of salinity and P is highly dependent upon the plant species (or cultivar), plant developmental age, the composition and level of salinity and the concentration of P in the substrate. Salinity-induced reduction in P uptake was observed by many workers in different crops such as Akbar (1975) in rice, Roberts *et al.* (1984) in maize.

Calcium plays an important role in processes that preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport and selectivity and control ion-exchange behaviour as well as cell wall

enzyme activities (Rengel 1992; Marschner 1995). The primary effect of salt stress is the disruption of membrane integrity caused by the displacement of Ca^{2+} from the surface by Na^+ (Cramer *et al.* 1985; Lynch *et al.* 1987; Suresh *et al.* 1991). Cramer *et al.* (1987) showed the evidence for displacement of membrane-associated Ca^{2+} by Na^+ in root hairs of salinized cotton (*Gossypium hirsutum* L.) seedlings. They found that high concentration of Na^+ displaced Ca^{2+} from plasma membrane. Salinity-induced reduction in calcium uptake was observed by Nakamura *et al.* (1990) in mungbean, Patil *et al.* (1992; 1995) in greengram. Increased NaCl induces decrease in Ca^{2+} levels in a number of plants (Al-Zaharani and Hajar 1998; Khan *et al.* 1999; 2000a; Khan 2001).

In the reported study, the content of Na^+ in root and leaf was higher in NaCl treatments than control. However, the content of Na^+ in leaf was significantly higher in SS2 than Pusa Jai Kisan at both NaCl levels. Maximum root Cl^- content was found with 100 mM NaCl in both the cultivars. At both NaCl levels the content of root Cl^- was higher in Pusa Jai Kisan than SS2. In contrast, leaf Cl^- content was higher in SS2 than Pusa Jai Kisan at both the NaCl levels. The treatment of 100 mM NaCl did not significantly enhance root and leaf Na^+ and leaf Cl^- content in both the cultivars over 50 mM NaCl (Figure 8). The difference in sodium absorption capacity of plants has been reported in maize (Yeo *et al.* 1977), rice (Yeo and Flowers 1984) and wheat (Ralph and Epstein 1986). Increased concentration of NaCl increased Na^+ and Cl^- in plants (Gadallah 1999). Salinity stress affects water availability due to the limitation of water uptake of plants, and excessive uptake of Na^+ and Cl^- results in limited assimilation of mineral nutrients (Serrano *et al.* 1999; Keutgen and Pawelzik 2009). In my experiment, the treatment of NaCl caused greater decrease in leaf water potential and higher accumulation of Na^+ and Cl^- in leaf in SS2 than Pusa Jai Kisan causing greater loss in turgidity in SS2. Kim *et al.* (2004) have shown that differential accumulation of Na^+ and Cl^- in root and leaf of plants grown under salt stress determines lipid peroxidation. There exists a direct relationship of accumulation of Na^+ and Cl^- in root and leaf with salt tolerance in the cultivars. The cultivar Pusa Jai Kisan exhibited high capacity of accumulating Na^+ and Cl^- in root than leaf, and in contrast SS2 showed higher content of Na^+ and Cl^- in leaf with lower content in root. The higher level of Na^+ and Cl^- in leaf

in SS2 induced greater oxidative stress affecting membrane permeability more adversely than in Pusa Jai Kisan causing greater reductions in photosynthesis and plant dry mass in SS2. The application of 1mM $SO4^{2-}$ to Pusa Jai Kisan and SS2 cultivars in NaCl stressed plants lowered the leaf Na^+ and Cl^- and root Na^+ and Cl^- at both the growth stages. There was further decrease in the accumulation of leaf Na^+ and Cl^- and root Na^+ and Cl^- with the application of 2 mM SO_4^{2-} to NaCl-treated plants. However, 2 mM SO_4^{2-} application to non-salinized plants did not prove beneficial to plants but in presence of NaCl it is a better dose in overcoming the accumulation of ions. Hence, Pusa Jai Kisan cultivar with high ATP-sulfurylase activity showed greater tolerance to salinity stress as a result of its capacity to accumulate Na^+ and Cl^- in root. From number of reports it is evident that salt stress causes nutrients deficiency due to competition of Na^+ and Cl^- with nutrients such as K^+, Ca^{2+}, $H_2PO_4^-$ and NO_3^- (Grattan and Grieve 1999; Maathuis and Amtmann 1999; Subbarao *et al.* 2003; Tester and Davenport 2003; Hu and Schmidhalter 2005). Availability of nutrients to plants depends on the activity of membrane transporters that translocate minerals from soil into the plant and mediates their intra- and inter-cellular distribution (Marschner 1995; Tester and Davenport 2003; Epstein and Bloom 2005; Akram *et al.* 2009). Increasing evidences suggest that mineral nutrients status of plants plays an important role in increasing plant resistance to abiotic stress.

Hence, application of 2 mM SO_4^{2-} treatment to salinity-treated plants had significant effect in increasing the contents of N, P, K and Ca and lowering the accumulation of Na^+ and Cl^- ions in comparison to their respective controls. The S application improves the soil structure and thus increases the usefulness of other nutrients to plants. It significantly increases the Cu, Mn, Ca and Mg contents of the shoot and grain of *Hordeum vulgare* L. (Togay *et al.* 2008). Sulfur interactions with other nutrients are directly related to the modification of physiological and yield responses of crops. This aspect of sulfur interaction with other nutrients requires in depth study. This would help to understand nutritional behaviour of S in relation to other nutrients and might provide guidelines for formulating balanced fertilizer recommendations in order to optimize yield and quality of produce.

5.3.5 Oxidative stress

Both osmotic and ionic effects involved in NaCl salinity can limit photosynthesis and respiration leading to an increase in ROS generation. Salinity stress impairs photosystems and Rubisco activity resulting in the imbalance in redox state of cell and generation of ROS (Figure 71).

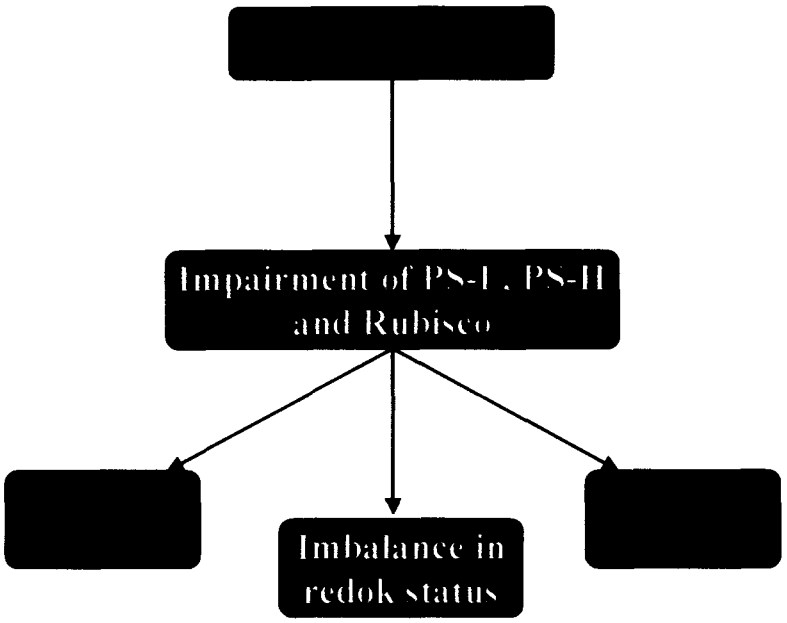

Figure 71. Salinity stress impairs redox status of cell and causes generation of ROS.

ROS are product of altered chloroplast and mitochondrial metabolism during stress, which are responsible for a secondary oxidative stress that can damage different cellular components including membrane lipids, proteins and nucleic acids. In the present study, NaCl-induced oxidative stress in *Brassica juncea* cultivars is evident from the significant increase in TBARS, H_2O_2 contents and electrolyte leakage with increasing NaCl levels. However, compared to the low ATP-sulfurylase activity cultivar SS2, high ATP-sulfurylase activity cultivar Pusa Jai Kisan showed significantly lower values for TBARS and H_2O_2 content and electrolyte leakage with increasing NaCl levels which are indicative of lower oxidative stress in Pusa Jai Kisan. The increase of about 3.5 times in TBARS and 2.5 times in H_2O_2 was observed in SS2 with the treatment of 100 mM NaCl compared to control, whereas in Pusa Jai Kisan, a lesser increase of 2.5 times in TBARS and 1.7 times in H_2O_2 was noted. Malondialdehyde is a product of cell membrane lipid peroxidation (Hegedus *et al.*

2001; Shah *et al.* 2001), which enhances on exposure of plants to some kinds of abiotic stress such as salinity, Zn and Cd toxicity (Chaoui *et al.* 1997a, b; Wu *et al.* 2003; Shao *et al.* 2008). Sairam *et al.* (2005) reported increase in TBARS and H_2O_2 content with the increase in salinity stress in *Triticum aestivum* genotypes and the increase was more pronounced in sensitive genotype. Salt treatment increases the content of H_2O_2 and peroxidation of the lipid membrane, thus disrupting its permeability or inducing oxidative stress in plant tissues (Gomez *et al.* 1999; Hernandez *et al.* 2000; Jain *et al.* 2001; Demiral and Turkan 2005; Mandhania *et al.* 2006). In addition, photorespiration is another consequence of low chloroplastic CO_2/O_2 ratio that tends to increase the cellular level of ROS such as H_2O_2 (Hernandez *et al.* 2000). Several plant species such as rice (Dionsio-Sese and Tobita 1998), wheat (Meneguzzo *et al.* 1999), pea (Hernańdez *et al.* 2000) and tomato (Sgherri *et al.* 2007; 2008) have shown to suffer oxidative stress under salt treatment. Other evidences also suggest that membrane injury under salt stress is related to the increased production of highly toxic ROS (Fadzilla *et al.* 1997; Gomez *et al.* 1999; Hernandez *et al.* 2000).

The enhanced cellular damage in SS2 seems to reflect the deterioration on the equilibrium between generation of ROS and defense mechanism towards removal of ROS. The addition of 2 mM SO_4^{2-} to NaCl-treated plants significantly and maximally reduced the contents of TBARS and H_2O_2 and electrolyte leakage. These results indicated that S application resulted in the amelioration of NaCl toxicity and protects photosynthetic apparatus of mustard plants. The positive effect of S in lowering the contents of TBARS and H_2O_2 and leakage of electrolytes was expected as ascorbate and glutathione production with S application helped to scavenge TBARS and H_2O_2 in both cultivars, but to a larger extent in Pusa Jai Kisan. The role of GSH in H_2O_2 scavenging in plant cells has been well established (Tausz *et al.* 2004; Srivalli and Khanna-Chopra 2008; Szalai *et al.* 2009). Glutathione takes part in the removal of excess H_2O_2 (Noctor and Foyer 1998) and lipid peroxides keeping ROS under control (Rausch *et al.* 2007). Glutathione reductase catalyzes the rate-limiting last step of asorbate-glutathione cycle and maintains high ratio of GSH to GSSG (Noctor and Foyer 1998). Thus, enhanced activity of ATP-sulfurylase and glutathione reductase and glutathione content resulted in lower content of TBARS and H_2O_2 and electrolyte

leakage in Pusa Jai Kisan than SS2 which resulted in lesser reduction in plant growth and photosynthesis.

Premachandra *et al.* (1990) reported that cell membrane stability is an indicator of stress tolerance. Membrane stability/injury has also been related with the tolerance or susceptibility of the various crop plants (Sairam *et al.* 1990; Sairam 1994; Kraus *et al.* 1995; Turhan *et al.* 2008). In the present study, membrane stability index decreased with NaCl concentration, while relative salt injury increased significantly. Pusa Jai Kisan showed significantly higher membrane stability index and lower relative salt injury compared to SS2. The increase in membrane stability index might be related to induction of antioxidant responses that protected the plant from oxidative damage. These results on the significant increase in membrane stability index are in conformity with the findings of Azooz (2009) in *Hibiscus sabdariffa* L.

Sulfur supplementation to NaCl-treated plants further increased the membrane stability index in both the cultivars and maximum significant increase was noted when grown with 2 mM SO_4^{2-} at both the growth stages. Application of 1 mM SO_4^{2-} alone (0 mM NaCl) increased the membrane stability index in both the cultivars, while 2 mM SO_4^{2-} reduced it. The reduction was less compared to 100 mM NaCl alone in both the cultivars. Supplementation of 1 mM SO_4^{2-} alone decreased relative salt injury with the increase in NaCl concentration in both the cultivars. Sulfur supplementation (2 mM SO_4^{2-}) to NaCl-treated plants lowered the relative stress injury while 1 mM SO_4^{2-} proved less effective.

5.3.6 Enzymatic antioxidants

Redox reactions play prominent roles in cellular responses to environmental stress. Biochemically, the metabolism of cells under stress is generally characterized by an increased formation of ROS (Elstner and Osswald 1994; Foyer and Noctor 2000). Stress-dependent ROS formation may also contribute to sensing and signaling of environmental stress impacts on plants. Even under non-stress conditions, the presence of ROS in cells is an inescapable feature of life in an oxygen atmosphere and serves as a signal molecule (Elstner and Osswald 1994). Plants have evolved antioxidant enzymes defense systems to keep ROS under control. The antioxidant enzymes are SOD, CAT, APX and GR. In fact, the expression of antioxidant enzymes is altered

under stress conditions. Their up-regulation has a key role in combating the abiotic stress-induced oxidative stress. A close association of photosynthetic electron transport and antioxidant enzymes exists for the removal of ROS and maintenance of redox state of cell (Figure 72).

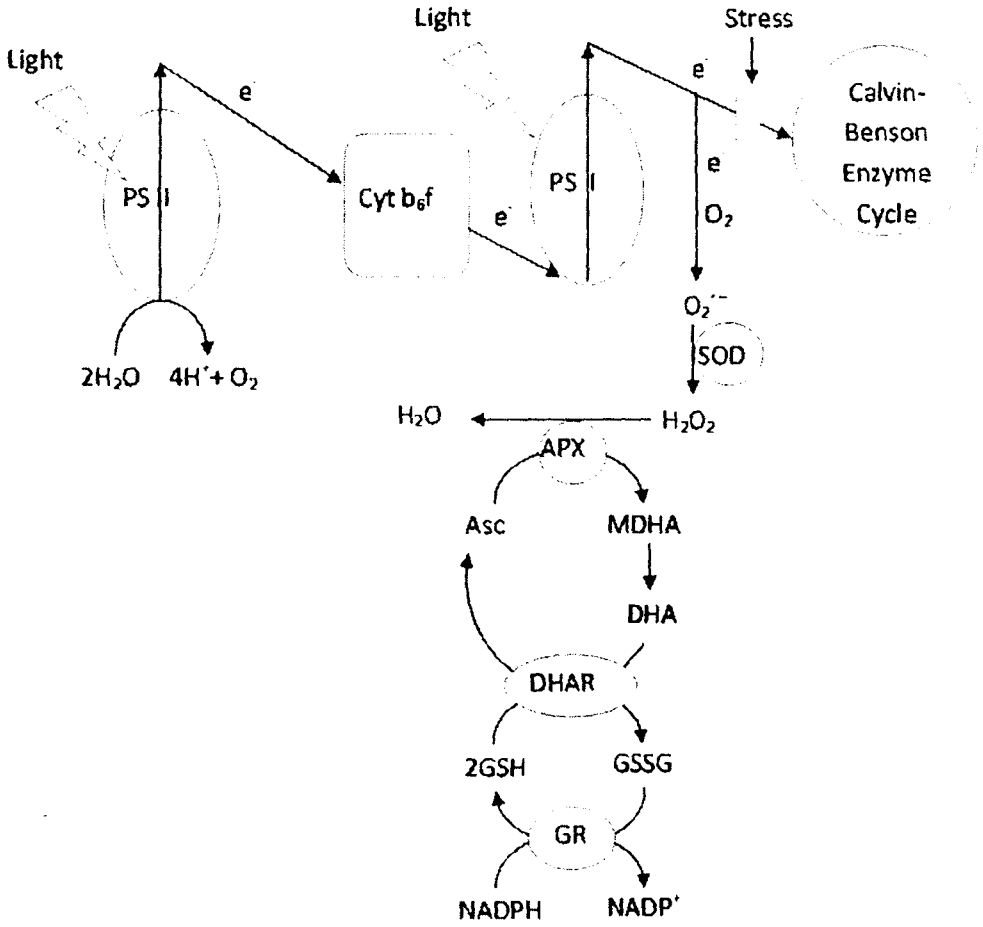

Figure 72. An association of photosynthetic electron transport and ascorbate-glutathione cycle for the removal of ROS

In the present investigation, the activity of antioxidant enzymes, SOD, CAT, APX and GR were enhanced in the presence of 50 and 100 mM NaCl. The activity of SOD was greater in SS2 than Pusa Jai Kisan at both the levels. In contrast, the activity of CAT, APX and GR was higher in Pusa Jai Kisan than SS2 and increased with NaCl compared to control in both the cultivars (Figures 27-30). Maximum activity was found with 100 mM NaCl.

Mobin and Khan (2007) also reported that higher SOD activity in *Brassica juncea* cv. RH30 was responsible for increased cellular damage due to excessive

accumulation of H_2O_2. It has been suggested that high SOD activity may be harmful for plants due to high H_2O_2 production, which in turn might inhibit other enzymes such as APX and CAT (Asada 1994; Agrawal and Mishra 2009). Hernandez *et al.* (2000) reported that APX, GR and SOD increased in wheat under salinity stress. In wheat, Meneguzzo *et al.* (1999) found differential antioxidative enzymes activity in shoot and root. In my study, the higher activity of SOD, which catalyzes the conversion of the superoxide anion to H_2O_2 and O_2 resulted in increased H_2O_2 content and greater damage to the membranes and the photosynthetic apparatus in SS2. Similar increases in the activity of SOD enzymes have been reported in cotton (Meloni *et al.* 2003), in *Cassia angustifolia* (Agarwal and Pandey 2004), *Beta maritima* and *B. vulgaris* cv. Ansa (Bor *et al.* 2003) and in maize (Tuna *et al.* 2008) subjected to salt stress. The higher activity of SOD, CAT, APX and GR has been observed in tomato plants under NaCl stress (Rodriguez-Rosales *et al.* 1999). However, some contradictory results are also available in the literature for SOD activity under saline conditions. For example, salt stress has been found to reduce SOD activity in pea (Hernandez *et al.* 1995) and rice (Dionisio-Sese and Tobita 1998).

Sulfur supplementation to Pusa Jai Kisan and SS2 treated with 100 mM NaCl lowered the SOD activity. The application of 2 mM SO_4^{2-} and 1 mM SO_4^{2-} lowered the NaCl-induced increase in SOD activity. The decrease in SOD activity is due to the less formation of ROS. The function of S in alleviating the stress may be attributed to its participation in the synthesis of GSH, an important ROS scavenger. Sulfur helped the plants to maintain high CO_2 (whose absence is the major cause of $O_2^{\cdot -}$ production in the process of photosynthesis), major reductants (ascorbate, glutathione) in chloroplasts and maintenance of the sulf-hydryl status of proteins. These processes diminished the chances of $O_2^{\cdot -}$ formation and its effects.

Catalase activity increased with the increasing NaCl concentration in both the cultivars but was higher in Pusa Jai Kisan than SS2 (Figure 18). The inhibition of CAT activity in SS2 may be due to its inactivation by excess H_2O_2 produced by SOD under NaCl stress. Agrawal and Rathore (2007) also reported that the decline in CAT activity was due to more consumption of CAT to detoxify H_2O_2 or its inactivation. The variable response of CAT activity has been observed under NaCl stress in different plant

species. For example, CAT activity increased in cotton (*Gossypium hirsutum*) when subjected to salt stress in salt-tolerant cultivars, whereas it remained unchanged or decreased in the salt-sensitive cultivars (Gossett *et al.* 1994, 1996). Costa *et al.* (2005) reported that CAT activity increased in leaves and roots of sorghum genotypes differing in salt tolerance, but the increase was higher in the salt-tolerant genotype.

Sulfur supplementation further increased the NaCl-induced increase in CAT activity at both the stages. In Pusa Jai Kisan, the increase in CAT activity due to 100 mM NaCl was further increased with the application of 2 mM SO_4^{2-} while 1 mM SO_4^{2-} increased the CAT activity to some extent. In contrast, 1 mM SO_4^{2-} application to non-salinized plants proved more beneficial compared to 2 mM SO_4^{2-} (Figure 6). Higher dose of 2 mM SO_4^{2-} application alone was harmful to non-salinized plants but when given to salt treated plants it helped in combating NaCl stress.

The activity of APX was found increased with 50 mM and 100 mM NaCl in both the cultivars and the activity was greater in Pusa Jai Kisan than SS2. Ascorbate peroxidase has higher affinity for H_2O_2 than CAT or peroxidase (Wang *et al.* 1999) and it has more crucial role in the management of ROS during stress. It is said to be responsible for the fine modulation of ROS for signaling. Gobinathan *et al.* (2009) reported that APX activity increased with 100 mM NaCl in *Pennisetum typhoides*. Enhanced activity of APX was also found in salt stressed *Raphanus staivus* plants (Lopez *et al.* 1996). The role of APX in stress tolerance has been shown in APX-antisense tobacco, a plant species highly sensitive to oxidative damage (Orva and Ellis 1997). Reports have shown enhanced expression of APX in plants in response to different abiotic stresses such as drought, salinity, high light intensity, SO_2 and O_3 (Tanaka *et al.* 1985; Mittler and Zilinskas 1992; Hernandez *et al.* 1995; Noctor and Foyer 1998). Furthermore, over-expression of APX in tobacco chloroplasts enhanced plant tolerance to salt and water deficit (Badawi *et al.* 2004). Considerable variations in the production of antioxidants, both enzymatic and non-enzymatic, in response to salt stress are evident at inter-specific or intra-specific level. For example, while examining the long-term effects of salt stress in two salt tolerant lines (Kharchia65, KRL19) and two salt sensitive lines (HD2009, HD2687) of wheat, Sairam *et al.* (2005) found that the salt tolerant line Kharchia65 showed less decline in the contents of ascorbic acid,

lower increase in H_2O_2 and TBARS and higher increase in SOD, APX and GR as compared to the salt sensitive line HD2687. In another study with the same crop, Mandhania *et al.* (2006) found that as compared to a salt tolerant line KRL19, a salt sensitive line WH542 suffered greater damage to cellular membranes due to lipid peroxidation as indicated by higher accumulation of H_2O_2, MDA and greater leakage of electrolytes. However, an almost uniform increase in the activity of CAT, APX and GR was observed with the increase in salt stress in both wheat cultivars. A recent study on the role of exogenous application of ascorbic acid on salt stressed wheat plants has shown the association of endogenous ascorbic acid level and activity of CAT with the salt tolerance of two lines differing in salinity tolerance (Athar *et al.* 2008). Comparing a salt tolerant maize line BR5033 with a salt sensitive BR5011 with respect to production of ROS and antioxidants, Neto *et al.* (2006) reported that salt-stressed plants leaves of both cultivars showed increased activity of SOD, APX, GPX and GR with time as compared to the non-stressed ones. However, the increase in the activity of antioxidant enzymes was more marked in the salt-tolerant cultivar than the salt-sensitive one. Additionally, the activity of SOD and CAT decreased, whereas those of APX, GPX and GR remained unaltered in salt-stressed roots of the salt-tolerant maize cultivar in comparison to the control.

Sulfur supplementation further increased the APX activity in both the cultivars. A combination of 100 mM NaCl and 2 mM SO_4^{2-} proved best in enhancing the APX activity. Thus, S alleviated the NaCl stress and helped plants to continue the APX activity to maintain the conversion of H_2O_2 into H_2O and O_2 by regenerating ascorbate and glutathione efficiently.

Glutathione reductase maintains the balance between GSH and ascorbate pools which in turn maintain cellular redox state (Lascano *et al.* 1999, 2001; Ansel *et al.* 2006; Romero-Puertas *et al.* 2006). In the reported study, the GR activity increased with the increasing NaCl concentration in both the cultivars and was higher in Pusa Jai Kisan than SS2. Increased GR activity facilitates improved stress tolerance and has the ability to alter the redox poise of the important components of the electron transport chain (Tyystjärvi *et al.* 1999). The increase in GR activity has been reported during water stress (Pastori and Trippi 1993). The increase in the GR activity has been linked

with the increased synthesis of protein under stress (Edwards *et al.* 1994). Overexpression of GR in chloroplasts conferred increased antioxidant protection to cold-induced photo inhibition and maintained the reduced ascorbate pool (Foyer *et al.* 1995). Similarly, increased GR activity during salinity stress has been reported in pea (Hernandez *et al.* 1993, 1995, 2000), cantaloupe (Fahmy *et al.* 1998), citrus (Gueta-Dahan *et al.* 1997), soybean (Comba *et al.* 1998), rice (Dionisio-See and Tobita 1998; Lin and Kao 2001; Vaidyanathan *et al.* 2003; Demiral and Turkan 2005; Tsai *et al.* 2005), *Cicer arietinum* (Kukreja *et al.* 2005), tomato (Shalata *et al.* 2001; Molina *et al.* 2002; Mittova *et al.* 2003), *Arabidopsis thaliana* (Huang *et al.* 2005), wheat (Sairam *et al.* 2005) and *Vigna radiata* (Sumithra *et al.* 2006).

Sulfur treatment increased the GR activity in both the cultivars. Maximum increase in GR activity was noted in Pusa Jai Kisan. The increase in GR activity due to the combined treatment 2 mM SO_4^{2-} and 100 mM NaCl was more when compared with the treatment of 100 mM NaCl alone. The up-regulation of GR activity may result in the improvement of abiotic stress tolerance by reducing GSSG produced in ascorbate-glutathione cycle. Besides, S plays a crucial role in the synthesis of cysteine, a precursor molecule for the production of GSH (Suter *et al.* 2000). Glutathione reductase catalyses the NADPH-dependent reaction of disulphide bond of GSSG and thus important for maintaining the reduced pool of glutathione. In the plants, the majority of the H_2O_2 generated during stress may be scavenged by the ascorbate-glutathione cycle, and GR has a central role in the H_2O_2 scavenging cycle in plants growing under environmental stresses.

It is suggested that the higher efficiency of the antioxidative system in the salt-tolerant genotype may be considered as one of the factors responsible for its tolerance to salt stress.

5.3.7 Non-enzymatic antioxidants

The ascorbate-glutathione cycle is present in almost all the compartments tested so far and that includes chloroplast, cytosol, mitochondria, peroxisomes and apoplast (Jimenez *et al.* 1997; Mittova *et al.* 2000), which suggests the importance of this cycle in controlling the level of ROS. Glutathione as non-enzymatic antioxidant plays a protective role in stress tolerance and acts as an important signal molecule directly

linking environmental stress and key adaptive responses (May *et al.* 1998a). Increased glutathione redox state may serve as a signal affecting the expression of defense genes (Khan *et al.* 2002). The changes in the processes regulating GSH concentration and/or redox status are considered to be one of the important adaptive mechanisms of plant exposed to stress conditions (Fadzilla *et al.* 1997; Alscher *et al.* 2002). Ascorbate is another major antioxidant and has been detected in majority of plant cell types, organelles and apoplast (Noctor and Foyer 1998; Horemans *et al.* 2000; Smirnoff 2000). It normally occurs in reduced form (AsA) (90% of the ascorbate pool).

In the present study, NaCl significantly decreased the reduced ascorbate content at both the stages and the decrease was higher in low ATP-sulfurylase cultivar SS2 than high ATP-sulfurylase cultivar Pusa Jai Kisan (Figure3l). Ascorbate together with glutathione affects plant tolerance to ROS by participation in the detoxification of ROS in plant cells (Noctor and Foyer 1998; Wu and Zhang 2002). Ascorbate peroxidase activity, in the present investigation, was found enhanced in both the cultivars. Maximum utilization of ascorbate by up-regulation of APX activity might be one of the major reasons of the reduction in ascorbate content. Qadir *et al.* (2004) and Anjum *et al.* (2008c) also reported that APX activity efficiently converts H_2O_2 into H_2O_2 and O_2 molecules using ascorbic acid as a reductant.

The application of 2 mM SO_4^{2-} under non-saline condition increased the reduced ascorbate content in both the cultivars significantly. Application of 2 mM SO_4^{2-} on plants treated with 100 mM NaCl increased the reduced ascorbate content, while 1 mM SO_4^{2-} alone which is beneficial to plants, when given to plants treated with 100 mM NaCl lowered the reduced ascorbate content in both the cultivars. In the present study, the improved reduced ascorbate content by S application probably reacted with $O_2^{\cdot -}$, $_1O_2$ (directly), H_2O_2 (enzymatically through APX) and thereby assisted in maintaining these potential toxicants in lower limits. In defense strategies against abiotic stress, reduced ascorbate is considered as a crucial component in photosynthetic tissues (Noctor and Foyer 1998). Reduced ascorbate eliminates ROS through coordination with the production of GSH in ascorbate-glutathione cycle (Foyer and Halliwell 1976; Noctor *et al.* 2002).

Glutathione is a tripeptide (γ-Glutamylcysteinylglycine) which occurs predominantly in the reduced form (GSH). GSH plays a central role in several physiological processes, including regulation of sulfur transport, signal transduction, conjugation of metabolites and detoxification of xenobiotics (Xiang *et al.* 2001). In addition, GSH is a substrate for glutathione peroxidase (GPX) and glutathione-S-transferase (GST), which are also involved in the removal of ROS (Noctor *et al.* 2002). GSH is a precursor of phytochelatins, which are important in controlling cellular heavy metal concentrations. GSH and its oxidized form, GSSG maintains a redox balance in the cellular compartments. This property of glutathione is of great biological importance since it allows fine-tuning of the cellular redox environment under normal conditions and upon onset of stress and provides the basis for GSH stress signaling. GSH can also directly reacts with $^1O_2$, $O_2^{\cdot -}$, and $^{\cdot}OH$. This reductive potential of GSH is due to the central nucleophilic cysteine residue. It may also stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation.

The content of GSH was found higher in Pusa Jai Kisan than SS2 and increased significantly with the increasing NaCl concentrations. Maximum GSH content was found with 100 mM NaCl treatment in both the cultivars. Ashraf and Harris (2004) showed that high levels of antioxidants and an active ascorbate-glutathione cycle are associated with salt tolerance in cotton. It is also known to regulate expression of certain stress defense genes during environmental stress (Ball *et al.* 2004) such as drought (Herbinger *et al.* 2002, Tausz *et al.* 2004), salinity (Vaidyanathan *et al.* 2003), chilling (Kocsy *et al.* 2000, 2001) and ozone (Noctor *et al.* 2002).

Sulfur application further increased the GSH content of both the cultivars with greatest increase in its content was noted in plants grown with 2 mM SO_4^{2-}. The association between lower TBARS level with S application under NaCl has shown the role of S in the alleviation of NaCl-induced oxidative stress. S is required for the synthesis of various compounds, such as thiols (GSH), sulpholipids and secondary S compounds, which play an important role in the metabolism of plants and in the protection and adaptation of plants against stress. The role of GSH in the H_2O_2 scavenging in plant cells has been well established (Tausz *et al.* 2004; Srivalli and Khanna-Chopra, 2008; Szalai *et al.* 2009). Glutathione takes part in the removal of

excess H_2O_2 (Noctor and Foyer, 1998) and lipid peroxides keeping ROS under control (Rausch *et al.* 2007). Earlier, we have reported that higher expression of ATP-sulfurylase activity is necessary for the maintenance of optimal GSH levels required for the proper functioning of ascorbate-glutathione cycle (Khan *et al.* 2009a, b). Further, the increased content of GSH with S application has been found to protect dry mass and photosynthesis of cadmium-treated mustard (*Brassica campestris*) (Anjum *et al.* 2008a). The content of the secondary S compounds is strongly dependent on the stage of development of the plant, temperature, water availability, and the level of S and N nutrition (Randle *et al.* 1993, 1995; Randle 2000; Randle and Lancaster 2002; Coolong and Randle 2003a, b). Fitzgerald *et al.* (1999) reported that plants develop substantial reserves of sulfate in the root and GSH in the leaves when they receive adequate S and N. Glutathione performs critical functions in regulating plant growth and adaptation to abiotic stresses and also acts as an important S sink in the plant system (Leustek *et al.* 2000, Maughan and Foyer 2006). The synthesis of GSH is mainly regulated by the availability of its constituent amino acids cysteine, glutamine and glycine and transcriptional regulation of enzymes of glutathione biosynthesis, γ-glutamylcysteine synthase and GSH synthetase (Tomaszewska 2002). Further, the S supplementation might help plants to improve the content of GSH by enhancing γ-ECS enzymes as shown by Schneider and Bergmann (1995) and Strohm *et al.* (1995). Based on the relationships between AsA and GSH pools with net photosynthesis and plant dry mass with and without S, Anjum *et al.* (2008a) suggested that adequate S supply might improve the pools of these compounds in plants to a great extent that led to the increase in photosynthetic efficiency and subsequently to plant dry mass and crop yield.

5.3.8 Growth characteristics

Plant growth and development are an outcome of coordination of several biological processes in plants (Vassilev *et al.* 1998). Plant growth and development are susceptible to stresses of all kinds including that of salinity stress. Salinity stress adversely affects plant growth and metabolism. The most common effect of NaCl toxicity in plants is stunted growth (Hernandez *et al.* 1995; Cherian *et al.* 1999; Takemura *et al.* 2000). Salt stress also results in a considerable decrease in the fresh and dry weights of leaves, stems, and roots (Hernandez *et al.* 1995; AliDinar *et al.*

1999; Chartzoulakis and Klapaki 2000). In the present study, growth decreased significantly with 100 mM NaCl in high and low ATP-sulfurylase activity cultivars, but more conspicuous in SS2 than Pusa Jai Kisan. Increasing salinity effects have been associated with significant reductions in shoot weight, plant height, and number of leaves per plant, root length, and root surface area per plant in tomato (Mohammad *et al.* 1998) and in cotton (Meloni *et al.* 2001). Moreover, the adverse effects of salinity on plant growth have been related to the decrease in osmotic potential of the growth medium, specific ion toxicity, nutritional imbalance and reduction in enzymatic and photosynthetic efficiency and other physiological disorders (Sultana *et al.* 1999; Demetriou *et al.* 2007). Application of 100 mM NaCl decreased the relative growth rate and was more pronounced in SS2 than Pusa Jai Kisan cultivar. The relative growth rate value reflects the life-sustaining activities of the plant, and is considered as an optimum index for degree of stress and plant responses to stress. The relative growth rate decreases under salt stress. Analyzing the effect of salinity stress on many plant species it may be concluded that NaCl reduces the relative growth rate through inhibiting mainly net assimilation rate and also by restricting leaf area ratio.

In Experiment 3, supplementation of 2 mM SO_4^{2-} to salinity-treated plants completely ameliorated the NaCl-induced toxicity and improved the growth characteristics of Pusa Jai Kisan and SS2. However, application of 1 mM SO_4^{2-} only lowered the reductions in plant dry mass and leaf area when applied to NaCl-treated plants. Under non-saline conditions the application of 1 mM SO_4^{2-} alone was beneficial to the crop, but 2 mM SO_4^{2-} proved inhibitory to both leaf area and plant dry mass in both the cultivars and the reductions were maximum in SS2 than Pusa Jai Kisan.

Under normal conditions the rate of uptake and assimilation of S is in line with the S requirement of plants for growth (De Kok *et al.* 2000). The S requirement for growth may vary during ontogeny of plants that largely differs between species. Sulfur requirement (equivalent to sulfur flux) of different crop species ranges from 2 to 10 μmol/g plant fresh weight/day under optimal growth conditions. In the present study, the application of S was found instrumental in mitigating the toxic effects of NaCl on plant growth characteristics. This indicates that S application reduces the toxic effects of NaCl on plants through improvement in the tolerance capacity of the plant. S

supplementation to plants has been shown to result in greater biomass production under normal conditions (Ahmad *et al.* 2005; Khan *et al.* 2005).

5.3.9 Yield characteristics

Yield is the final manifestation of growth, photosynthesis and biochemical traits of a plant which are strongly regulated by several environmental factors. In our study, NaCl stress significantly decreased the yield characteristics and the extent of decrease was greater in SS2 than Pusa Jai Kisan. In line with the photosynthetic and growth characteristics, yield and its attributes were also decreased in mustard cultivars with NaCl treatment. Maximum reduction in yield and attributing traits was noted with 100 mM NaCl in both the cultivars. It is generally believed that abiotic stresses are the main source of yield reduction (Boyer 1982; Rehman *et al.* 2005; Munns and Tester 2008; Reynolds and Tuberosa 2008). The estimated potential yield losses are 17% due to drought, 20% due to salinity, 40% due to high temperature, 15% due to low temperature and 8% by other factors (Rehman *et al.* 2005; Ashraf *et al.* 2008; Athar and Ashraf 2009). In the present study, salt stress induces a reduction in chlorophyll content, affects photosynthetic electron transport and inhibits PSII activity as a consequence of the accumulation of salts in chloroplasts. This reduction in photosynthetic activity has been found directly related to the reduction in yield as observed by others (Meloni *et al.* 2003; López-Climent *et al.* 2008). Therefore, the inhibition of photosynthetic activity, decreased biomass production, higher accumulation of Na^+ and Cl- ions and lower content of mineral nutrients lead to the reduction in final yield of the crop.

Munns and Rawson (1999) observed that 100 to 175 mM NaCl showed a significant reduction in spikelets per spike, delayed spike emergence and reduced fertility and poor wheat grain yield. Rice plants died before maturity with the increase in salinity level beyond 100 mM NaCl. Barley, the most tolerant cereal died after extended periods at salt concentration higher than 250 mM NaCl (Maas and Hoffman 1977; Maas and Grattan 1999; Munns *et al.* 2006; Chen *et al.* 2009).

The reduction in yield characteristics due to 100 mM NaCl in my study was completely ameliorated by 2 mM SO_4^{2-} application in NaCl-treated plants, whereas application of 1 mM SO_4^{2-} lowered the NaCl-induced stress effects in both the

cultivars. In non-salinized plants, application of 1 mM SO_4^{2-} significantly increased the yield characteristics in both the cultivars but was found higher in Pusa Jai Kisan. However, 2 mM SO_4^{2-} was found less effective. The improvement in yield characteristics due to S application to NaCl-fed plants can be correlated with the enhancement in high ATP-sulfurylase activity, growth, photosynthesis, and biochemical characteristics. Furthermore, S application reduced the Na^+ and Cl^- uptake, H_2O_2 and TBARS content along with significant increase in enzymatic and non-enzymatic components of antioxidant defense system which in turn reduced the NaCl-induced oxidative stress and hence provided tolerance to plants and maintained the yield.

A model is given to show the influence of salinity stress-induced changes in plants decreasing growth and yield, and how sulfur restores the decrease caused by salinity stress (Figure 73).

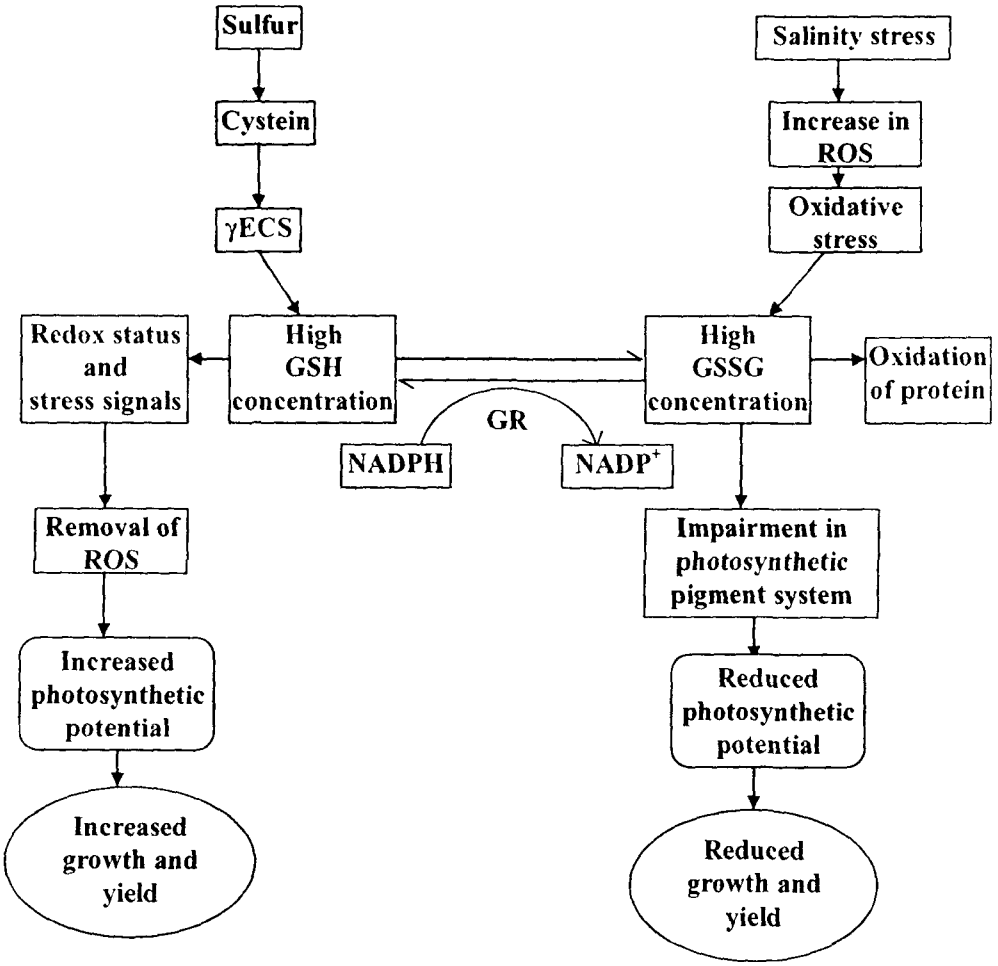

Figure 73. Salinity stress-induced effects on growth and yield and the influence of sulfur in the alleviation of salinity-induced effects.

Sulfur is an essential macronutrient that plays a vital role in the regulation of plant growth and development (Ernst 1998; Anjum *et al.* 2008a, b; Nazar *et al.* 2008) in higher plants, and S limitation results in decreased yields and quality parameters of crops (Hawkesford 2000). Glutathione content of plant tissues is an indicator of S nutritional status of plant (Blake-Kalff *et al.* 2000). Indeed GSH content of young maize seedlings is drastically reduced when plants are sulfur-deprived (Petrucco *et al.* 1996; Bolchi *et al* 1999; Quaggiotti *et al.* 2003).

5.4 Conclusion

Conclusively, it may be said that S is one of the critical factors determining the photosynthesis, growth and yield of plants under normal and salinity stress conditions. However, in recent years widespread deficiency of S in soil of crop fields has been noticed in many parts of India, and one of the reasons for the low status of S in soil is leaching (Tandon 1991). Mustard has high demand for S, and is particularly sensitive to S-deficiency. It produces seeds with high content of S rich proteins (Zhao *et al.* 1997; Blake-Kalff *et al.* 1998). There have been studies in relation to S application and its effects on metabolism of various plants as emphasized in 'Review of Literature'. However, the study on the application of S to NaCl-treated high and low ATP-sulfurylase activity mustard types and its effect on growth and metabolism have not been reported. As discussed in the previous pages the adequate supply of S improved photosynthesis, growth and yield through its effect on the control of nutrients and ions accumulation, water relations, oxidative stress and enzymatic and non-enzymatic antioxidants. Its application also alleviated NaCl-induced decrease in photosynthesis, growth and yield. The response of S application was higher in high ATP-sulfurylase activity cultivar Pusa Jai Kisan than low ATP-sulfurylase activity cultivar SS2. The application of S on sulfur assimilation, photosynthetic traits, water relations, content of nutrients and ions, oxidative stress, various components of antioxidant defense system and growth and yield characteristics of high and low ATP-sulfurylase mustard types under NaCl stress has been reported for the first time. In this study efforts have been made to develop S protocol for the alleviation of NaCl stress in mustard and to

elucidate how ATP-sulfurylase activity determines growth, photosynthesis and yield of NaCl-grown plants.

At molecular level, stress response proteins are transcripted with the supply of sulfur. An effort has been made in preparing a model presented as Figure 74 to show the assimilation of S in chloroplast and production of stress response proteins.

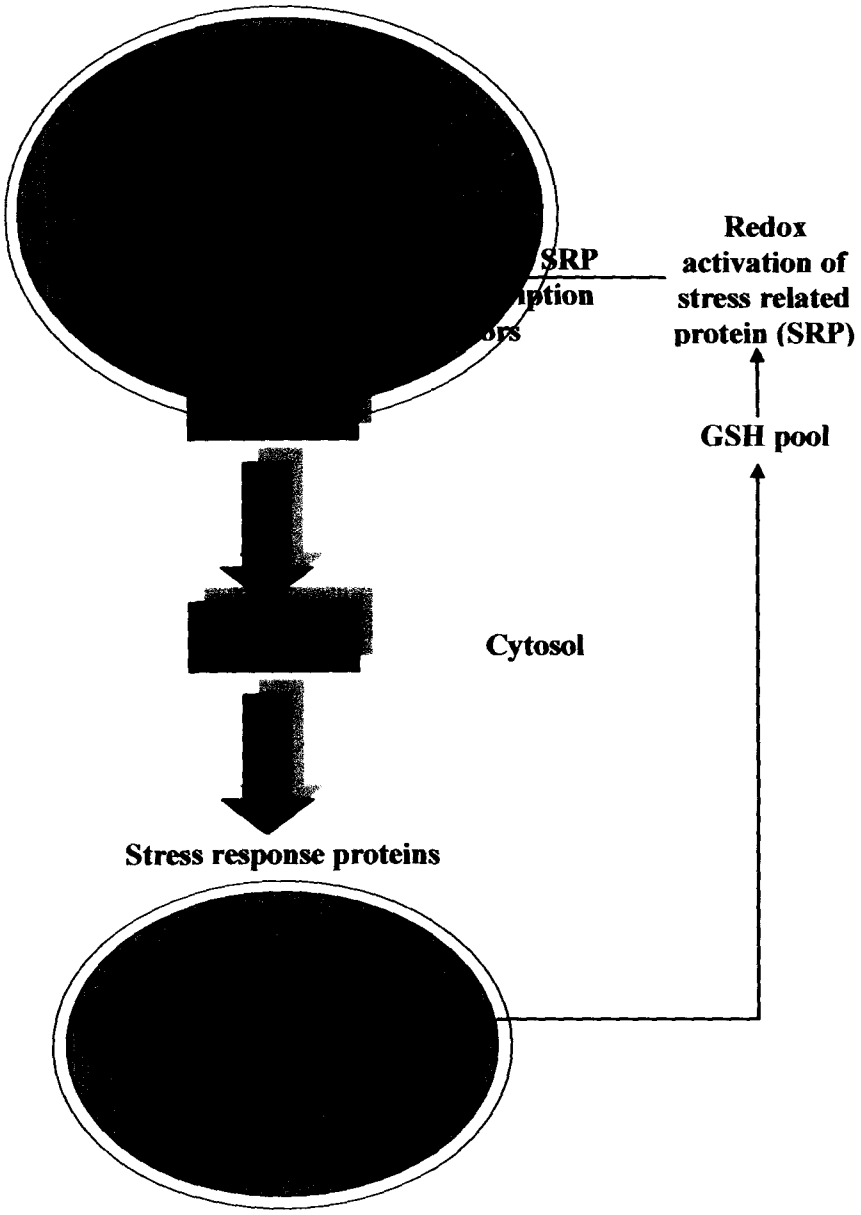

Figure 74. Assimilation of sulphur helps in the transcription of stress response proteins.

5.5 Future Research Prospects

Salinity is the major environmental factor limiting plant growth and productivity in the arid and semi-arid regions of the world. The continuous

accumulation of salt in cultivated soils as a result of poor irrigation and climate warming increases the importance of the study of this stress factor. Salinity stress causes in osmotic inhibition and ionic toxicity, which affects the physiological and biochemical functions of plant cell. The present study showed that maximum reduction in the growth, photosynthetic, biochemical and yield characteristics was with 100 mM NaCl in both the cultivars of mustard, i.e., Pusa Jai Kisan and SS2. However, the reduction in the characteristics was much pronounced in SS2. Mineral nutrient status plays an important role in increasing plant resistance to environmental stress factors. Of the mineral nutrients, S constitutes one of the macronutrient necessary for the plant life cycle. The processes of the uptake and assimilation of S in higher plants are crucial factors determining plant growth and vigor, crop yield and the resistance to biotic and abiotic stresses. Sulfur assimilates not only play key roles in the primary metabolism of plants and provide structural components of essential cellular molecules, but also act as signaling molecules for cellular communication with the environment.

Future strategies are focused to modulate steps of S assimilation pathways leading to the production of thiols and their products in plants through manipulating serine acetyl transferase (SAT), γ-glutamyl- cysteine synthetase and glutathione synthetase enzymes under salinity stress. The carbon backbone of glutathione is provided by serine which is converted to O-acetyl serine by serine acetyl transferase. O-acetyl serine is then converted to cysteine and glutathione. Thus, serine acetyl transferase may execute a central ole in glutathione synthesis and control of oxidative stress under salinity stress. It is likely that an increase in the serine acetyl transferase activity could increase tolerance of plants to conditions which cause oxidative damage. More detailed studies are required to understand the NaCl-induced stress response modulated by S metabolism at physiological and molecular levels. This would have dual advantage of correcting S deficiency and resistance to salinity stress. The transgenic may be tailored to have high ATP-sulfurylase activity for sustainable plant productivity under salinity stress.

Summary

SUMMARY

The present thesis entitled "Physiological Significance of Sulfur in Growth and Metabolism of Mustard (*Brassica juncea*) Exposed to Salinity Stress" comprises of six chapters.

In Chapter 1 (Introduction) describes the importance of the problem and justifications for the present work undertaken were explained.

Chapter 2 is the Review of Literature. It deals with the relevant literature on the aspects of salinity stress and the importance of S nutrition in the alleviation of salinity stress on various crop plants. The chapter has been divided into sections and sub-sections for better understanding of the work of other research workers reported in this field of study.

Chapter 3 (Material and Methods) gives details of the materials used in the study and methodology adopted to determine various physiological, biochemical, growth and yield characteristics recorded in the experiments. In addition, relevant information on the plant sampling and experimental design has been mentioned.

Chapter 4 (Results) includes details of the three experiments. Variation among mustard cultivars for ATP-sulfurylase activity and sulfur accumulation capacity were recorded. Details of physiological and biochemical processes occurring in low and high sulfur accumulation capacity cultivars under salinity stress and response of these two cultivars to sulfur application alone or in combination with salinity stress have been included. The data were statistically analyzed and level of significance was determined at $P<0.05$ using analysis of variance (ANOVA).

In Chapter 5, results have been discussed in the light of observations recorded and supported with the earlier findings, if available on the subject. Possible explanations of the data obtained have also been included to reach a conclusion and possible future prospects.

Chapter 6 presents the summary of the work reported in this thesis.

Experiment 1

Experiment 1 was conducted on four mustard (*Brassica juncea* L.) cultivars namely, Alankar, Varuna, Pusa Jai Kisan and SS2 to select S-efficient and S-inefficient cultivars on the basis of ATP-sulfurylase activity. In addition, sulphate content (SO_4^{2-})

in root and leaf, N content in leaf, growth, photosynthetic characteristics at 30 and 60 DAS were also recorded. Sulfate transport index (STI) was calculated as the ratio of sulfate content in root and leaf and expressed as percentage. The relationship of ATP-sulfurylase activity with photosynthetic rate and shoot dry mass was also established. The treatments were arranged in a randomized block design and replicated three times. All the cultivars differed in ATP-sulfurylase activity and sulfate transport index. Pusa Jai Kisan showed maximum ATP-sulfurylase activity and STI followed by Alankar, Varuna and SS2. Leaf sulfate, nitrogen content, photosynthetic rate, leaf area and shoot dry mass were also increased from 30 to 60 DAS. A strong positive correlation ($P<0.01$) between ATP-sulfurylase activity and photosynthetic rate and shoot dry mass was found in all the four cultivars. It was concluded that the activity of ATP-sulfurylase may be used as a physiological trait for augmenting photosynthesis and shoot dry mass accumulation in mustard.

Experiment 2

Experiment 2 was conducted on the basis of findings of Experiment 1. As observed in Experiment 1, Pusa Jai Kisan emerged as S-efficient and SS2 as S-inefficient mustard cultivars. This experiment was conducted with the aim of studying the influence of 0, 50 and 100 mM NaCl on sulfur assimilation, photosynthetic traits, water relations, contents of nutrients and ions, oxidative stress, various components of antioxidant defense system and growth in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (low ATP-sulfurylase activity) cultivars of mustard at 30 and 60 DAS and yield characteristics at 120 DAS. The experiment was conducted in a factorial randomized block design and each treatment was replicated three times. Maximum reductions in the growth, photosynthetic characteristics and nutrients content were noted with 100 mM NaCl at 30 and 60 DAS in both the cultivars of the mustard. Plants treated with 100 mM NaCl exhibited a significant and maximum decrease in the characteristics over control. The activity of ATP-sulfurylase in plants grown with NaCl was significantly higher than the control in both the cultivars. Pusa Jai Kisan exhibited higher ATP-sulfurylase activity than SS2 at both NaCl levels. However, the effect of 50 and 100 mM NaCl on ATP-sulfurylase activity did not differ significantly in both the cultivars. The cultivar Pusa Jai Kisan exhibited high capacity of accumulating Na^+ and

Cl^- in root than leaf. Contrarily, SS2 showed higher content of Na^+ and Cl^- in leaf with lower content in root. The higher level of Na^+ and Cl^- in leaf in SS2 induced greater oxidative stress affecting membrane permeability more adversely than in Pusa Jai Kisan causing greater reductions in photosynthesis and plant dry mass. Pusa Jai Kisan exhibited lower induction of superoxide dismutase activity but higher induction of catalase, ascorbate peroxidase and glutathione reductase in comparison to SS2. The lower superoxide dismutase activity in Pusa Jai Kisan was due to the lower content of leaf Na^+ and Cl^-. The enhanced activity of ATP-sulfurylase and glutathione reductase and glutathione content resulted in lower content of TBARS and H_2O_2 in Pusa Jai Kisan. This cumulatively resulted in lesser reductions in photosynthetic functions in Pusa Jai Kisan. The cultivar Pusa Jai Kisan with high ATP-sulfurylase activity showed greater tolerance to salinity stress as the result of its capacity to accumulate Na^+ and Cl^- in root, higher water and osmotic potential, efficient antioxidant system and higher glutathione content. The increased activity of antioxidant enzymes and glutathione in this cultivar removed ROS more efficiently. These characteristics of Pusa Jai Kisan helped in protecting the photosynthetic capacity and maintaining high plant dry mass. Contrarily, SS2 cultivar with low ATP-sulfurylase activity had higher accumulation of Na^+ and Cl^- in leaf, lower water potential and osmotic potential, lower contents of nutrients, greater oxidative stress and poor capacity of antioxidant system resulting in lower photosynthesis and dry mass than Pusa Jai Kisan.

Experiment 3

Experiment 3 was conducted to study the influence of 1 or 2 mM SO_4^{2-} in the alleviation of 100 mM salinity stress in Pusa Jai Kisan (high ATP-sulfurylase activity) and SS2 (low ATP-sulfurylase activity). Treatment of 100 mM NaCl, 1 mM SO_4^{2-} or 2 mM SO_4^{2-} was given alone or in combination to Pusa Jai Kisan and SS2 to observe their response on sulfur assimilation, photosynthetic traits, water relations, contents of nutrients and ions, oxidative stress, various components of antioxidant defense system and growth at 30 and 60 DAS, and yield characteristics at harvest. The treatments were arranged in a factorial randomized block design and each treatment was replicated three times.

Salinity stress led to a significant reduction in photosynthetic traits, water potential and osmotic potential, nutrients content and yield characteristics of both the cultivars. The low ATP-sulfurylase activity cultivar SS2 exhibited a higher reduction than high ATP-sulfurylase activity cultivar Pusa Jai Kisan. In comparison to 1 mM SO_4^{2-}, the application of 2 mM SO_4^{2-} maximally alleviated the adverse effects of 100 mM NaCl and improved the photosynthetic traits, water and osmotic potential, nutrients content, components of enzymatic and non-enzymatic antioxidant system, growth and yield characteristics of both the cultivars. In general and under non-stressed condition, application of 1 mM SO_4^{2-} instead of 2 mM SO_4^{2-} was found positive in improving photosynthesis, water relations, plant growth, antioxidant metabolism, growth and yield in both the cultivars. However, when applied in combination with 100 mM NaCl, higher dose of S (2 mM SO_4^{2-}) was found effective in alleviating the 100 mM NaCl-caused effects in both the cultivars but to a great extent in high ATP-sulfurylase activity cultivar Pusa Jai Kisan compared to low ATP-sulfurylase activity cultivar SS2. It is pertinent to mention here that the application of sulfur either alone or in combination with 100 mM NaCl further increased the ATP-sulfurylase activity and S content in both the cultivars, which were found greatest in Pusa Jai Kisan than SS2. The supplementation of 100 mM NaCl-treated plants with SO_4^{2-} (1 or 2 mM) further enhanced the NaCl-induced increase in the activity of catalase, ascorbate peroxidase and glutathione reductase at both the growth stages in both the cultivars.

The present chapter is followed by an up-to-date bibliography of the literature cited in the text.

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